THE BRITISH JOURNAL OF EXPERIMENTAL PATHOLOGY

VOL. XXVI DECEMBER, 1945 NO. 6

EXPERIMENTAL PRODUCTION OF URATE CALCULI IN THE URINARY TRACT OF WHITE RATS.

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Received for publication October 10, 1945.

URATE calculi have hitherto received relatively little attention in the literature dealing with the experimental production of urinary concretions.

Ebstein and Nikolaier (1891) found uric acid infarcts, but never observed calculi in experimental animals which had received injections of piperazine. Kleinschmidt (1911) mentioned casually the appearance of urate calculi in rams which had been fed special diets, the composition of which was not noted. Rost (1930) reported on urate calculi in rats receiving a vitamin A-free diet, to which a large amount of uric acid had been added. Mostly the concretions were not larger than " grains of sand," and could be detected in the urine only by microscopical examination. Randall, in 1940, reviewed the problem of the experimental production of urinary calculi. He stated that attempts at producing urinary calculi composed of uric acid or urates with the help of a vitamin A-free diet have never been successful. According to Keyser (1943), overfeeding of laboratory animals with uric acid never results in the formation of calculi.

The aforementioned negative results are understandable in view of the fact that in all mammals, with the exception of man, chimpanzee, and the Dalmatian coach dog, uric acid is oxidized by the liver to allantoin, a substance which does not form urinary concretions because of its high solubility in water (Rose, 1923).

Sedimentation of uric acid has, in fact, been observed in the bladder of the common dog and of monkeys after extirpation of the liver (Mann, 1944), and in the common dog also where an Eck fistula had been established (Fischler, 1925). In the latter instance true calculi composed of uric acid may form (Hagar and Magath, 1928). More recently Higgins (1943) tried to produce urate stones in two Dalmatian puppies by feeding them a diet deficient in vitamin A, and was successful in one animal.

In the following we report on experiments in which the formation of urate calculi in the urinary tract of rats was observed following damage of the liver by prolonged administration of carbon tetrachloride.

METHODS.

Forty-four male white rats were used; at the beginning of the experiments they weighed between 35 and 50 gm. The majority of animals received carbon tetrachloride "Analar" (British Drug Houses), and the remainder a commercial brand of this drug. Carbon tetrachloride diluted with an equal part of mineral oil was administered by subcutaneous injection twice weekly for 25 weeks. 0·1 c.c. carbon tetrachloride per 100 g. body weight was given at each injection.

The animals received a diet of wheat grain and a measured portion of milk; tap water was given *ad libitum*.

The rats were sacrificed at various times between 30 and 90 days following the fiftieth injection.

Eight rats succumbed in the 24th to 25th week of treatment, one to six days following their last injection. These cases are included in the report.

RESULTS.

(a) *Lesions of the Liver.*

Changes of varying character and intensity were found in the liver. In some of the animals the liver was of usual size, and showed grossly no pathological changes except small isolated superficial nodules (Group 1). In other cases only slight dimpling or discrete granulations of the surface was found (Group 2). In yet others the liver was firm in texture, and the surface of the organ was studded with innumerable tiny nodules of uniform size, each defined by a deep retraction of the surface revealing characteristics of fine annular cirrhosis (Group 3). There were also instances in which the liver was smaller than usual, and presented a surface deformed by nodular formations which were irregular in size, and separated from one another by fairly wide depressed areas presenting an aspect of coarsely-nodular cirrhosis (Group 4, Fig. 1).

The above described lesions of all types appeared with nearly equal frequency in the group of 8 rats which was examined from one to six days following the last injection, and in the main group of 36 rats which was examined 30 to 90 days after the last injection.

Histologically Groups 1 and 2 showed no significant difference. There was complete transformation of the acini. The latter were surrounded by delicate strands of connective tissue, which could only be identified in some cases by the use of special stains for collagen.

The liver in rats belonging to Groups 3 and 4 showed transformation of acini and extensive fibrosis. The latter was most marked in the central portions of the liver in both of the groups. In cases of coarsely-nodular cirrhosis the fibrotic areas sometimes extended over wide areas of the liver surface (Fig. 2).

(b) *Findings in the Urinary Tract.*

In 39 rats the lumen of the bladder as well as that of the ureters and renal pelvis was empty, and the renal pelvis was not dilated.

In the remaining 5 rats the urinary bladder contained 3–8 calculi, the diameter of which varied between 0·5 and 2 mm. (Fig. 3). In one of these rats smaller concretions of the same kind were found in the pelvis of one kidney.

The calculi were spherical in shape, smooth in surface and of a yellowish-brown colour throughout. They were quite hard in texture, and could not be crushed by hand between glass-slides. After mounting in Canada balsam they could be ground easily with fine sandpaper.

In chemical examination the calculi were found to be mainly composed of urates mixed with traces of ammonium-magnesium phosphate.

The ground disc of such a calculus shows a regular lamellar structure. Its concentric lamellae lie closely together. They are separated by discrete layers which can be stained with cresyl violet (Fig. 4).

(c) *Correlation of Findings in the Liver and in the Urinary Tract.*

TABLE I.

Liver lesion.	Number of animals.*	Number of animals with urinary calculi.
Grade 1	12 (2)	—
,, 2	10 (1)	—
,, 3	17 (4)	—
,, 4	5 (1)	5 (1)
	44 (8)	5

* The numerals in parentheses refer to the number of rats examined from 1 to 6 days after the last injection.

The development of urate calculi in these experiments was related to the presence of coarse-nodular cirrhosis, which in 4 out of 5 instances was still persistent from 30 to 90 days after the cessation of the treatment with carbon tetrachloride. Calculi were not found in rats having fine annular cirrhosis, but without extensive macroscopic transformation, or in rats whose liver displayed transformation of acini without accompanying increase of fibrous tissue.

DISCUSSION.

As is known from reports in the literature, prolonged treatment of rats with carbon tetrachloride eventually leads to the development of hepatic cirrhosis. However, it has been shown by Cameron and Karunaratne (1936) that changes so induced are highly reversible; within a fortnight following the last administration of the drug all macroscopic signs of the cirrhosis may disappear. The same authors observed that the cirrhotic changes may persist in animals which receive more than 40 injections of 0·1 c.c. of carbon tetrachloride if coarsely nodular cirrhosis develops. This is precisely the situation in our experiments.

The coincidence observed in our experiments between the occurrence of coarse nodular cirrhosis and the appearance of urate calculi raises the question whether restoration failure in a cirrhotic liver and excretion of urate instead of allantoin stem from a common cause.

Since histological study has failed to explain why "fine annular cirrhosis" is reversible, whereas coarse nodular lesion is permanent, any attempt at explaining our observations must be conjectural.

Cameron and Karunaratne discussed the possibility that coarse nodular cirrhosis becomes permanent owing to a "grave alteration in the regenerative capacity of the liver cell," which may be due to the "toxic agent itself or products of necrosis produced by it."

It is conceivable that the same alteration may interfere with the uricolytic function of the liver. Nevertheless, this explanation is not satisfying, since the activity of

toxic agents of the kind suggested by Cameron must reasonably be assumed to intervene in the earlier reversible as well as in the irreversible stages of liver lesion.

It seems to us to be more likely that restoration failure in coarsely nodular cirrhosis is inherent in the architectural transformation of the liver and its vascular apparatus in particular.

It has been shown that the regeneration of hepatic parenchyma is almost entirely dependent on the adequacy of the portal blood-flow (Mann, 1940). In man vascular injections reveal a gradual development in advanced cirrhosis of shunts, between the portal and the hepatic veins functionally analogous to an Eck fistula. The occurrence in our rats of a similar transformation of the vascular ramifications with subsequent reduction of supply of portal blood to the liver would explain the persistence in these animals of cirrhotic changes. Inhibition of uricolysis and excretion of urates in the urine could likewise probably result from the pre-hepatic deviation of portal blood-flow which accompanies the reorganization of vascular supply in the cirrhotic liver. On this view the formation of urate calculi in cases of coarse nodular cirrhosis becomes comparable to the urate sedimentation which occurs in the urinary bladder of dogs following the establishment of an Eck fistula.

SUMMARY.

Urinary calculi are shown to occur in male white rats which are treated with carbon tetrachloride for an extended period of time. The calculi are composed largely of urates.

This formation is correlated with the development of marked hepatic fibrosis and deformation (coarse nodular cirrhosis).

The coincidence of urate excretion with the occurrence of a certain type of hepatic lesion is discussed, and a possible explanation of the observed correlation is offered.

The author is indebted to Prof. E. Wertheimer for the chemical analysis of calculi.

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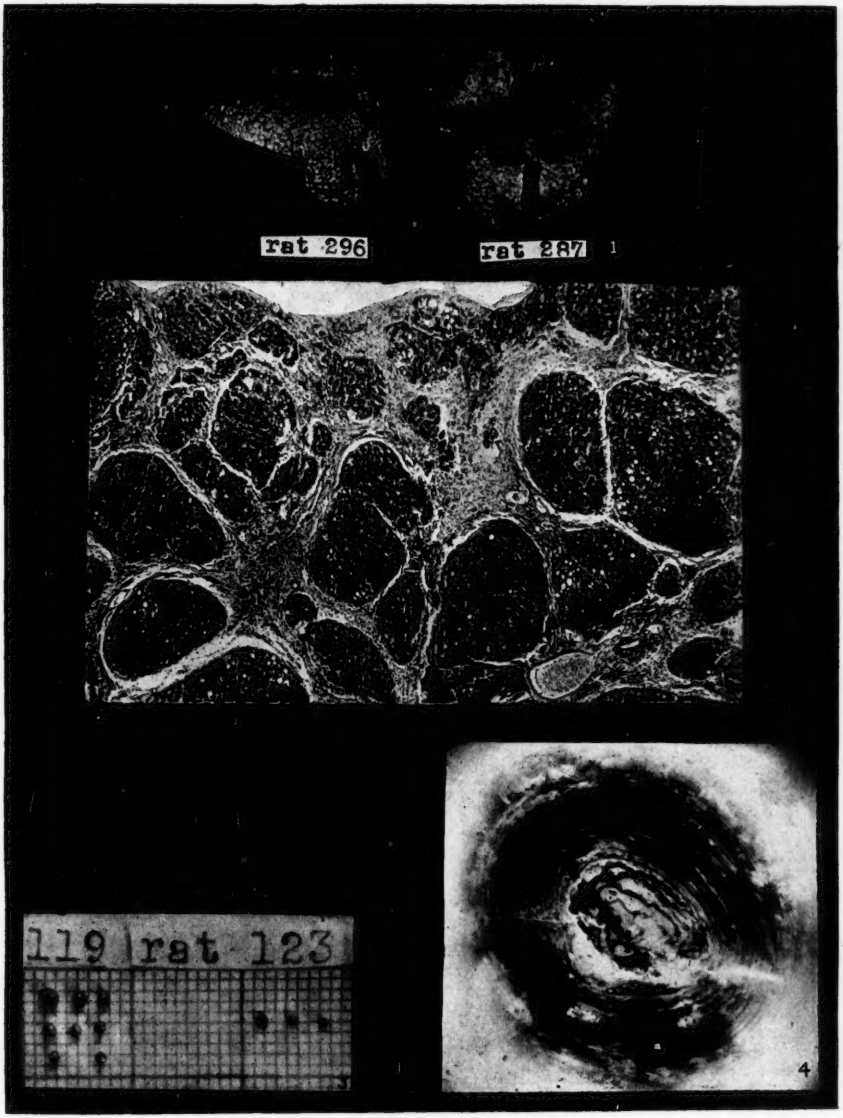

FIG. 1.—Liver from rats receiving 50 injections of 0·1 c.c. carbon tetrachloride twice weekly. Thirty days after last dose. On the left, fine annular cirrhosis: on the right, coarse nodular cirrhosis of animals with urate calculi.

FIG. 2.—Liver with coarse nodular cirrhosis.

FIG. 3.—Urate calculi from the bladder.

FIG. 4.—Ground disc from bladder calculus composed of urates; counterstained with cresyl violet. (× 42.)

Ungar.

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THE ENZYMES OF HEALING WOUNDS. II: THE EFFECT OF DIFFERENT DEGREES OF VITAMIN C-DEFICIENCY ON THE PHOSPHATASE ACTIVITY IN EXPERIMENTAL WOUNDS IN THE GUINEA-PIG.

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Received for publication October 12, 1945.

USING the histochemical method described by Takamatsu (1939) and Gomori (1939), Fell and Danielli (1943) showed that the regenerating connective tissue in healing skin wounds in the rat contained alkaline phosphatase, the activity of which increased as healing advanced, reached a maximum when collagen-formation was greatest, and declined during the later stages of repair to the low level normal for the adult skin. It was suggested that the enzyme might be concerned in collagen-formation.

Recently Biesele and Biesele (1944) observed phosphatase activity in the skin of mice during carcinogenesis produced by methylcholanthrene, and they also noted a phosphatase reaction, similar to that described by Fell and Danielli, in an accidental wound in one of their treated animals.

It has long been known that in vitamin C-deficiency wounds heal slowly and the scar when formed is liable to break down. Histological studies (reviewed by Bourne, 1942; Wolbach and Bessey, 1942) have shown that this is due to the "inability of the supporting tissues to produce and maintain intercellular substances" (Wolbach and Howe, 1926). The histological details of wound healing in C-deficiency have recently been clearly described by Hunt (1941). In view of the association between the appearance of phosphatase and the differentiation of fibrous tissue previously observed in the rat (Fell and Danielli, 1943), it seemed desirable to find whether vitamin C-deficiency affected the phosphatase activity in healing wounds as well as the regeneration of fibrous tissue.

Histochemical observations have already been made by Bourne (1943) on the phosphatase activity of regenerating skeletal tissue in normal and C-deficient guinea-pigs. Holes were bored in the femora and allowed partly to heal. In the non-scorbutic controls the cells and fibres of the regenerating tissue gave a strongly positive reaction with Gomori's method, but "microscopically there was scarcely any phosphatase reaction in the holes in the femora of the scorbutic animals." The alkaline phosphatase reaction of kidney and other tissues and organs in normal and vitamin C-deficient guinea-pigs has been investigated by Harrer and King (1941), and Russell, Rouse and Read (1944). Shwachman and Gould (1942*a*, *b*; 1943) found a lowered blood phosphatase in vitamin C-deficiency.

In our present study we have observed the effect of different degrees of vitamin C-deficiency on the phosphatase activity and differentiation of the scar tissue in small open skin wounds in guinea-pigs.

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EXPERIMENTAL.

Diet.

Guinea-pigs of both sexes and about 300 g. in weight were used. In Experiments 1 and 2 the effect of an acute vitamin C-deficiency was studied; in Experiments 3 and 4 the guinea-pigs were divided into groups supplied with various levels of vitamin C. Four animals died shortly after operation and were discarded.

The basal diet (Harris and Oliver, 1942) without vitamin C was composed of 720 g. oatmeal, 90 g. dried egg yolk, 160 g. bran, 20 g. salt mixture, 100 g. dried yeast, and 6 drops of radiostoleum weekly to supply vitamins A and D. When vitamin C was given it was prepared immediately before use and administered daily by mouth with a dropping pipette before feedings.

Experiment 1 (18.ii.43–11.iii.43).

Acute vitamin C-deficiency. Six guinea-pigs were fed on the basal diet without ascorbic acid and five controls were given 10 mg. ascorbic acid daily. Wounds (see below) were made on the 7th day of the experiment, and animals were killed 1, 3, 6, 9, 13 and 16 days later.

Experiment 2 (18.viii.43–16.ix.43).

Acute vitamin C-deficiency. Twelve animals were fed on the basal diet without vitamin C and 6 controls received 10 mg. ascorbic acid daily. The operations were done on the 6th day of the experiment. One, two or three deficient guinea-pigs were killed respectively on the 8th, 10th, 12th, 16th, 18th, 22nd and 24th day after operation, and one control was killed on the 8th, 12th, 22nd and 24th day.*

Experiment 3 (22.iii.44–30.v.44).

Partial vitamin C-deficiency; 0·5 mg. level. Nine animals were fed on a completely deficient diet for 10 days to deplete their reserves of vitamin C and were then given 0·5 mg. ascorbic acid daily. Six controls were dosed with 10 mg. ascorbic acid daily from the beginning of the experiment. The operation was performed on the 24th day of partial deficiency. Two, three or four animals were killed respectively on the 22nd, 28th and 36th day after operation, i.e. 46, 52 and 60 days after being put on 0·5 mg. ascorbic acid daily.

Experiment 4 (6.x.44–6.xii.44).

Partial vitamin C-deficiency; 0·5–10 mg. levels. Thirty-one guinea-pigs were divided into groups of 3–7 animals, and after a preliminary period of 9 days without vitamin C were dosed daily with 0·5 (7 animals), 0·7 (6 animals), 1·0 (4 animals), 2·0 (4 animals), 5·0 (3 animals), and 10 mg. ascorbic acid (4 animals). All the wounds were made on the 24th day of partial deficiency and the guinea-pigs were killed 28 days after operation (i.e. after 9 days of acute deficiency + 52 days of partial deficiency).

* Since the maximal phosphatase activity of wounds in normal guinea-pigs is not reached until about the 3rd week after operation, an attempt was made to prolong the life of a few of the acutely deficient animals by giving doses of vitamin C during the latter part of the experiment. One of the two animals killed after 18 days received two doses of 10 mg., the last dose 2 days before death, one of the animals killed after 22 days received 5 doses of 10 mg., the last dose 5 days before death, and both the animals killed after 24 days received one dose of 2 mg. 8 days before death. The condition of the animals and the histological picture of the scar seemed almost unaffected by the few doses given at this late stage of deficiency, although, as will be seen from Experiment 4, dosage with ascorbic acid after a shorter period of acute deficiency has a very pronounced effect.

To control the possible effect on healing of the 9-day period of acute deficiency, a group of 3 guinea-pigs received 10 mg. ascorbic acid from the beginning of the experiment; the operations were made at the same time as in the other groups, and the animals were killed 28 days after the operation.

Surgical Methods.

Standard wounds were made under ether anaesthesia by the method described by Dann, Glücksmann and Tansley (1941). An area of dorsal skin was closely shaved and wiped with 80 per cent alcohol. A circle was cut in this area with a rotating cylindrical blade 6 mm. in diameter, and the skin was excised with fine scissors. The wounds were not covered.

Histological Methods.

Immediately the guinea-pig had been killed, a flap of skin containing the wound was removed, spread on clean filter-paper, and with a safety razor blade cut in half across the transverse axis of the scar. One half, which was used to study the distribution of phosphatase, was fixed in 80 per cent alcohol, dehydrated in absolute alcohol, cleared in cedar wood oil, and infiltrated first in soft wax at 40° C. and then in hard wax at 58° C. The other half, which was fixed in 3 per cent acetic Zenker for 5 hours and then treated in the usual way, was used to investigate the histological structure of the scar by other methods. Sections were cut at 10μ.

As in our previous experiments (Fell and Danielli, 1943), the preparations made by Gomori's method (Gomori, 1939) were incubated in the glycerophosphate solution for 24 hours instead of 2–3 hours as originally prescribed by Gomori. Slides made from the Zenker-fixed material were stained by haematoxylin followed by chromatrop or van Gieson's stain, by a modification of Mallory's triple stain in which carmalum is substituted for the acid fuchsin, and by Wilder's silver method counterstained with carmalum and a mixture of light and naphthol green.

RESULTS.

1. *Clinical Observations.*

In acute deficiency the animals in both Experiments 1 and 2 lost weight from the 13th day onwards, and when the last were killed on the 24th day after operation they showed characteristic signs of scurvy, and a large scab still covered the incompletely epithelialized wounds.

In Experiment 3 (0·5 mg. ascorbic acid) the initial weight was maintained, but epithelialization was greatly delayed. The control wounds healed during the second week, but in the deficient animals even 4 and 5 weeks after operation the wounds were swollen and had a large scab and extensive adhesions. There were usually haemorrhages round the knee-joints and scapulae.

In Experiment 4 (see Table I) the gain in weight was related to the size of the dose almost up to the 10 mg. level (see Fig. 1 and 2), but there were not sufficient animals in each group for a statistical test of the significances of these differences to be made. The results, however, agree well with the clinical and histological findings described below. One animal in the 10 mg. group deprived of vitamin C for a preliminary period of 9 days behaved erratically, its weight curve failed to rise and it was discarded.

Healing proceeded normally only at the highest level, i.e. the 10 mg. level with and without a previous acute deficiency. In the whole experiment one wound only showed obvious signs of infection and was suppurating slightly.

TABLE I.—*Summary of Clinical and Histological Findings in Experiment 4.*

Dose of ascorbic acid mg./day (after 10 days' depletion).	Clinical.			Histological.	
	Rate of healing.	Adhesions.	Haemorrhages.	Phosphatase.	Differentiation.
0·5	±	++++	+	+	+
0·7	±	++++	+	+	+
1·0	+	++++	+	+	+
2·0	++	0	+	++	++
5·0	+++	0	0	++++	+++
10·0	+++	0	0	+++	++++
10·0*	++++	0	0	+++	++++

* This group was dosed with 10 mg. ascorbic acid daily without a previous acute deficiency of vitamin C.

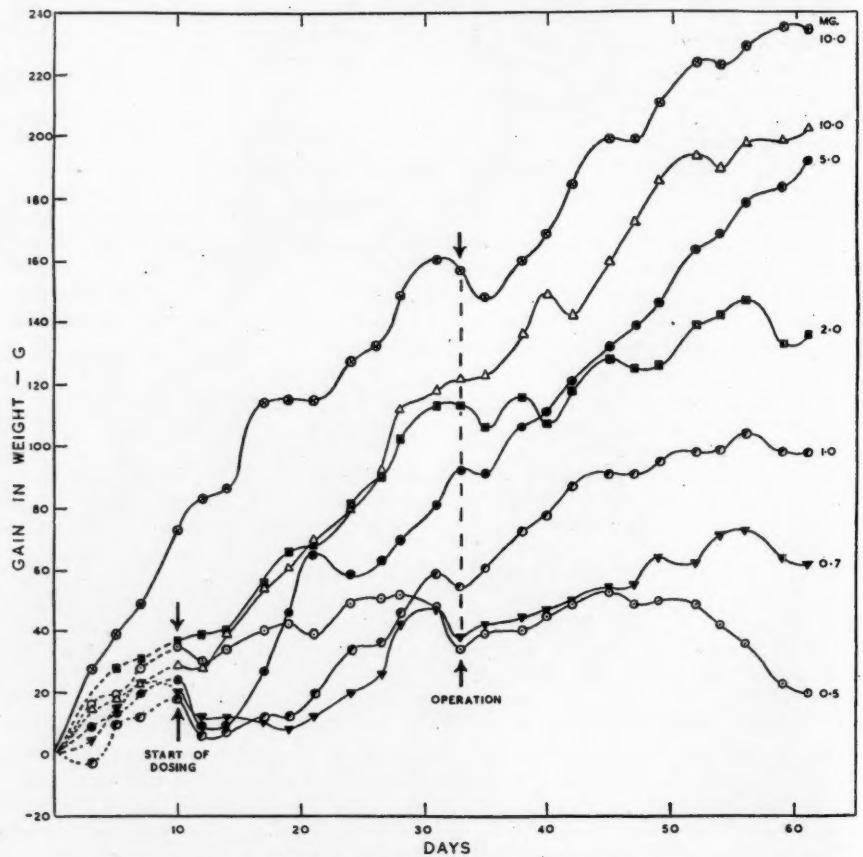

FIG. 1.—Weight gains with various intakes of vitamin C.

It can be seen that the rate of healing went parallel with the degree of phosphatase activity and of differentiation, but inversely to the formation of adhesions and haemorrhages in the scar tissue.

The wounds in the 10 mg. group without a preliminary acute deficiency were nearly healed two weeks after operation, and those in the other 10 mg. and in the 5 mg. groups were fully and normally healed after three weeks. At the lower levels of vitamin C, however, healing was still incomplete when the guinea-pigs were killed 28 days after operation ; at this stage the scars in the 0·5 and 0·7 mg. series showed large adhesions, occasional scabs and little or no hair at the site of injury. In the 1·0 mg. group the wounds were partially epithelialized, but displayed adhesions and haemorrhages, while at the 2 mg. level, though epithelialization was complete, haemorrhages were seen in about 50 per cent of the scars.

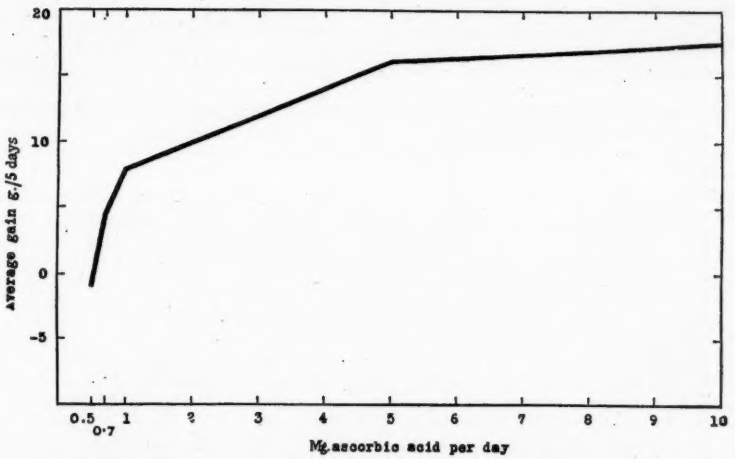

FIG. 2.—Average weight gains for ten 5-day periods throughout the experiment at different levels of vitamin C.

2. *Histological Observations.*

A. *Healing in the normal guinea-pig.*

In preparations of uninjured skin made by Gomori's method, the sebaceous glands, the proximal part of the hair follicles and a few capillaries give a strong positive reaction for phosphatase, but the rest of the skin and the subcutaneous tissue show almost no reaction.

Wounds from 18 control guinea-pigs belonging to all four experiments have been examined histologically, and the results are included in the following account of healing in the normal animal. The wounds were fixed at intervals of from 1 day to 5 weeks after operation.

In the 3-day wound (Fig. 3) the cavity is partly filled by an oedematous bulge of subcutaneous tissue which is densely infiltrated with leucocytes, mainly polymorphs, and covered by a scab. Gomori's method demonstrates an intense phosphatase activity both in the wound itself and in the interstices of the dermis and muscle, which is localized in the leucocytes and particularly in the polymorphs (*cf.* Gomori, 1943 ; Fell and Danielli, 1943). As in rat wounds, the scab with its very high content

of leucocytic debris is nearly black. The fibroblastic nuclei of the surrounding tissue also give a positive but weaker reaction.

The subcutaneous tissue plays a more important part in healing than is usual in similar wounds in the rat, and by the 6th day the subcutaneous bulge seen in the day-old wound is larger, richly vascularized and, except in the depths of the scar, is closely packed with fibroblasts orientated parallel with the surface of the w ound. Fibroblasts

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Fig. 3–15. Abbreviations: b., blood; b.p. blood pigment; mi., mitosis; o.d., original dermis; p.r., phosphatase reaction.

Fig. 3–9.—The preparations were made by Gomori's method from the half-wounds fixed in alcohol and were photographed at a magnification of × 18.

Fig. 3.—Normal guinea-pig; wound fixed 3 days after operation. At this stage almost all the phosphatase activity is due to the infiltrating leucocytes. In the subcutaneous tissue bulging into the cavity the reaction is slight except near the surface, but the scab, which contains much polymorph debris, is nearly black.

Fig. 4.—Normal guinea-pig; wound fixed 16 days after operation. Epithelialization is complete, and the second peak of phosphatase activity associated with the differentiation of fibrous tissue has begun to appear. The reaction is intense at the margin of the wound, but still only moderate in the interior.

Fig. 5.—Normal guinea-pig; wound fixed 21 days after operation. The phosphatase reaction in the scar is now maximal.

Fig. 6.—Acute vitamin C-deficiency; animal received one 2 mg. dose of ascorbic acid 8 days before death; wound fixed 24 days after operation. The scar gives no appreciable phosphatase reaction (the dark deposit in the centre is blood pigment).

Fig. 7.—Partial vitamin C-deficiency; 1·0 mg. level; wound fixed 28 days after operation. There is a slight phosphatase reaction in the deeper parts of the scar tissue. Both the scab and a small pocket of pus beneath it react intensely owing to the leucocytes present, but the superficial scar tissue below the pus gives no reaction (the apparent darkening is due to extravasated blood under the epidermis).

Fig. 8.—Partial vitamin C-deficiency: 2 mg. level. Wound fixed 28 days after operation. The scar is much more normal than that shown in Fig. 5. In the regenerating fibrous tissue the phosphatase reaction is intense immediately beneath the epidermis, but only moderate elsewhere.

Fig. 9.—Partial vitamin C-deficiency: 5 mg. level. Wound fixed 28 days after operation. The scar appears normal, is greatly contracted, and gives an intense phosphatase reaction throughout (*cf.* Fig. 3).

Fig. 10–15. The preparations were made from the half-wounds fixed in Zenker's solution and were photographed at a magnification of × 300. All the photographs are orientated in the same way, i.e. the top of each field is roughly parallel to the epidermis.

Fig. 10.—Normal guinea-pig; wound fixed 12 days after operation. Note the thin, regularly arranged argyrophil fibres. (Wilder's method; carmalum and light green mixture.)

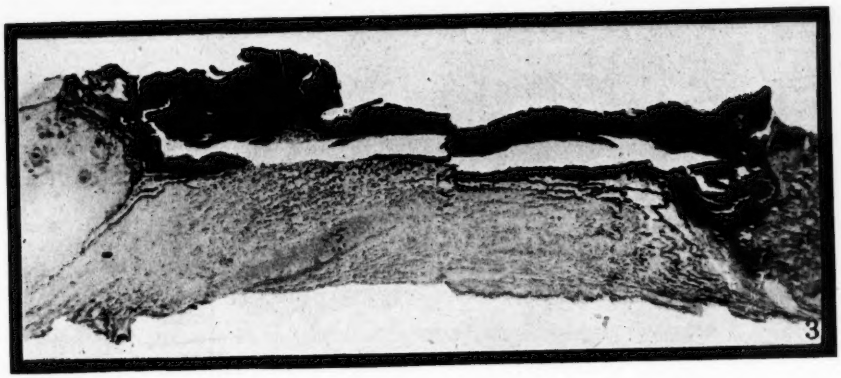
Fig. 11.—Acute vitamin C-deficiency; wound fixed 12 days after operation. Note the chaotic arrangement of the argyrophil fibres, some of which form stout irregular strands. (Wilder's method; carmalum and light green mixture.)

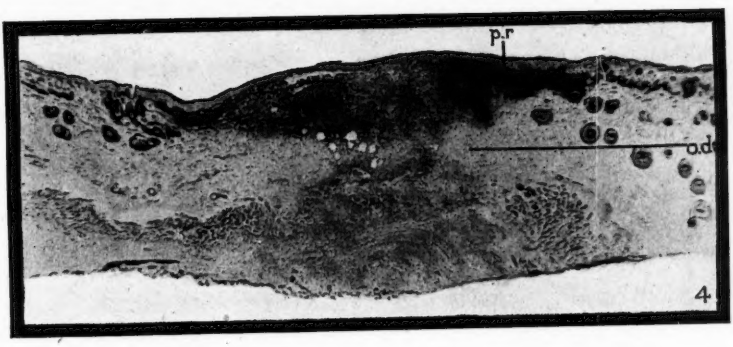
Fig. 12.—Acute vitamin C-deficiency; section of the same wound as that shown in Fig. 4, fixed 24 days after operation. Note the swollen irregular masses of abnormal collagen and the chaotic arrangement of the cells. (Haematoxylin and van Gieson's stain.)

Fig. 13.—Partial deficiency; 1 mg. level. Section of the same wound as that shown in Fig. 5, fixed 28 days after operation. The intercellular fibres, though relatively scanty, are much more normal than those shown in Fig. 10, but the arrangement of both cells and fibres is very irregular, and in this part of the scar they are orientated at right angles to the epidermis. (Haematoxylin and van Gieson's stain.)

Fig. 14.—Partial deficiency: 2 mg. level. Section of the same wound as that shown in Fig. 6, fixed 28 days after operation. The fibres, though rather fine, are nearly orientated parallel to the epidermis. (Haematoxylin and van Gieson's stain.)

Fig. 15.—Partial deficiency: 5 mg. level. Section of the same wound as that shown in Fig. 7, fixed 28 days after operation. Note the abundant, well-developed fibres and orderly arrangement of the tissue. (Haematoxylin and van Gieson's stain.)





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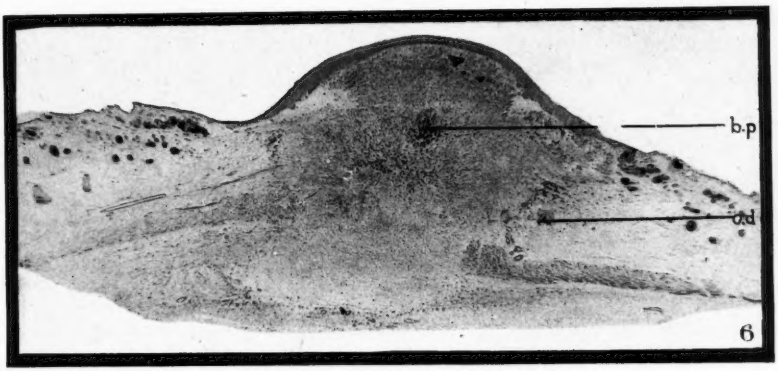

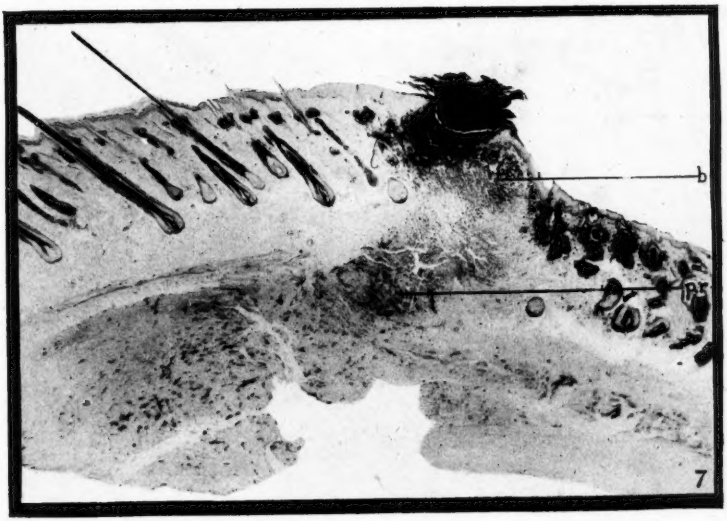

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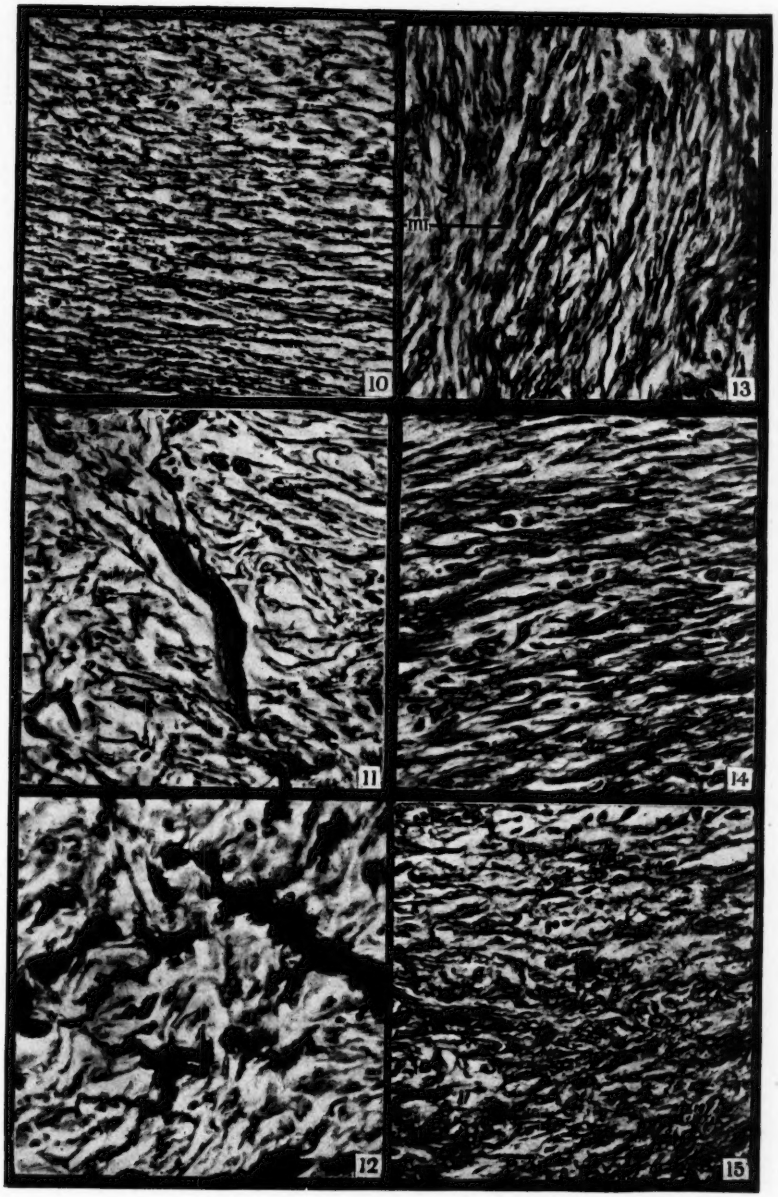

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have also begun to invade the wound from the cut edge of the dermis. Fine, wavy collagen fibres taking the characteristic blue colour with Mallory's stain have appeared between the cells, and are largest and most deeply stained in the tissue proliferating from the cut surface of the dermis. This dermal outgrowth gives a reaction for phosphatase, which is intense immediately beneath the growing edge of the epidermis, but elsewhere only moderate. In the central mass of the scar only a few capillaries and the invading leucocytes, now greatly reduced in number, react strongly, but some of the fibroblastic nuclei show slight activity.

As healing proceeds the dermal and subcutaneous outgrowths become indistinguishably welded and the wound slowly shrinks. The wavy collagenous fibres increase in number and thickness, and stain more deeply with aniline blue and less with silver than at earlier stages of repair. Fibre-formation continues to be most advanced near the cut edge of the old dermis and beneath the regenerating epithelium. The epithelium spreads completely over the wound, and new hair follicles and sebaceous glands begin to develop round the margin of the scar. A progressive increase in phosphatase activity accompanies these histological changes. By the 16th day (Fig. 4) the whole scar gives a positive reaction, which is most intense at the margin of the wound, and particularly in the space which intervenes between the regenerated epithelium and the young dermis, where both the cells and the growing fibres are nearly black.

The scar continues to contract, and the cut surfaces of the original dermis converge funnel-wise from the epidermis towards the bottom of the wound, where after 3–4 weeks the sides are nearly in contact. The parallel layers of abundant, fairly thick collagen fibres which now fill the cavity of the wound give an intense phosphatase reaction (Fig. 5), except in the much contracted region at the base of the scar, where the activity is only moderate.

In the two wounds fixed 5 weeks after operation the phosphatase reaction, though still pronounced, is less than at 4 weeks, and had probably begun to decline as in the fully healed rat wound (Fell and Danielli, 1943).

B. *Healing in acute vitamin C-deficiency* (Experiments 1 and 2).

The intact skin of the deficient animals differs little from that of the normal; the sebaceous glands and hair follicles both give a positive reaction for phosphatase.

Wounds from 17 guinea-pigs suffering from acute deficiency were examined histologically at intervals of from 1–24 days after operation.

As in the controls, the subcutaneous tissue bulges into the cavity of the deficient wound, and during the first day becomes invaded with leucocytes and covered by a scab. Both leucocytes and scab give an intense phosphatase reaction.

The small subcutaneous bulge seen in the 1–3-day wounds rapidly enlarges into a dome-shaped mass of haemorrhagic, oedematous tissue which is very poorly vascularized. The intercellular material consists entirely of a scanty, irregular argyrophil network of fine fibres and coarse fibrin-like strands composed of fine fibres embedded in amorphous material (Fig. 11). Some of the fibres, especially in the depths of the wound, stain blue like collagen with Mallory's method, but much of the fibrous network remains colourless or is coloured greenish orange. This intercellular material appears to be identical with the " precollagen " described by Hunt (1941) in C-deficient wounds. The orientation of both cells and fibres is chaotic (*cf.* Figs. 10 and 11), and there is very little outgrowth from the old dermis at any stage of healing. Only the scab and leucocytes give a strong phosphatase reaction and the regenerating connective tissue is

almost colourless, though there is sometimes a slight reaction round the proliferating hair follicles at the margin of the wound.

The histological character of the scar remains almost unchanged even 21–24 days after operation. The argyrophil network becomes more abundant than before, and the thick, irregular strands (Fig. 12), which are as plentiful as ever, assume a curious bloated appearance which makes them even more conspicuous than at an earlier stage. The scar still shows almost no phosphatase activity (Fig. 6). The much smaller, compact fibrous scars of the corresponding controls with their intense phosphatase reaction present a startling contrast to the vitamin C-deficient scars (*cf.* Figs. 5 and 6).

c. *The effect on healing of different levels of partial deficiency* (Experiments 3 and 4).

Observations were made on 16 guinea-pigs of the 0·5 mg. group, 2 of which were killed after 3 weeks, 10 after 1 month, and 4 after 5 weeks. The scar tissue closely resembles that of the acutely deficient animals except that the intercellular material is rather more abundant and less abnormal. After 4 and 5 weeks a few fine, wavy collagen fibres are present in addition to the irregular argyrophil network, and the thick strands, so conspicuous in acute deficiency, are much fewer and less swollen.

While some of the scars show very little phosphatase activity, others fixed after 4–5 weeks contain areas in which not only do the nuclei give a strong reaction, but the intercellular material also shows slight but definite activity. The tissue adjacent to the proliferating hair follicles and at the margin of the wound nearly always gives a reaction, which in some of the older wounds is intense and quite extensive. But although many of the wounds of the 0·5 mg. group give a stronger phosphatase reaction than we ever observed in acute deficiency, the reaction is nevertheless conspicuously weaker than at the highest levels of vitamin C, in which the fibrous scar is nearly black.

Wounds from 6 guinea-pigs of the 0·7 mg. group and 4 of the 1·0 mg. group were examined. All the animals of these and subsequent series were killed 28 days after operation. In histological appearance and phosphatase activity the scars of these groups (Figs. 7 and 13) show little improvement on the 0·5 mg. series.

The 4 scars of the 2·0 mg. group, however, are much nearer to the normal. They are smaller than those from the preceding groups, though usually larger than those from animals kept at the higher levels of vitamin C, the orientation of the fibroblasts is much more orderly than at the lower levels of vitamin C, and the intercellular fibres are more abundant and better differentiated (*cf.* Figs. 13 and 14) ; there is still some haemorrhage. The stout, irregular strands of precollagen seen in the previous series are absent. All four scars show considerable phosphatase activity (Fig. 8), which is intense immediately beneath the regenerated epidermis and rapidly diminishes towards the depths of the wound, where the nuclei only are dark and the intercellular material nearly colourless.

The three scars of the 5 mg. group are almost normal. As compared with those of the 2 mg. group they are more contracted, the intercellular fibres are thicker and more numerous, and phosphatase activity (*cf.* Figs. 8 and 9) is much more intense.

There was no significant histological difference between the four scars of the 10 mg. group and two which were examined from the group given 10 mg., but without a preliminary vitamin C-deficiency. As in the other series, there was some individual variation, but healing was advanced in all. The scars were much contracted, the cut surfaces of the old dermis being almost in contact at the base of some of the wounds, and were composed of well-developed fibrous tissue. The phosphatase reaction, though still intense in most of the scars, especially below the new epidermis, had probably passed its peak and was slightly less than in the 5 mg. group.

DISCUSSION.

In the normal guinea-pig, as in the rat, two peaks of phosphatase activity are seen during the healing of a wound, one due to the invading leucocytes, and a second which is associated with the differentiation of collagenous fibrous tissue. In vitamin C-deficiency the first peak is almost or quite as great as in the controls, but the size of the second peak depends on the degree of deficiency. Thus, in acute scurvy there is no second peak, and at different levels of partial deficiency the amount of phosphatase activity during the later stages of healing is correlated with the degree of differentiation of the scar. Our results show a marked contrast between the effect of 1 mg. of vitamin C per day, at which level differentiation is poor and phosphatase activity slight, and that of 2 mg., at which differentiation begins to approach the normal, and phosphatase activity, though much below that at higher levels of dosage, is considerable.

In our experiments the optimum level of vitamin C, as assessed by its beneficial action on rate of healing, on differentiation and on phosphatase activity, is not less than 5 mg., and possibly even higher. These findings agree well with the critical levels of vitamin C necessary for the repair of other types of tissue and for producing a scar of normal tensile strength (*cf.* Bourne, 1943, 1944; Hines, Larere, Thomson and Cretzmeyer, 1944; Kuether, Telford and Roe, 1944). The slightly lower level which some authors report as optimal is probably due to the fact that in our study the guinea-pigs were subjected to a preliminary period of acute deficiency to deplete their stores of vitamin C.

As regards the function of alkaline phosphatase in wound-healing, we suggested in our earlier paper " that the phosphatase is connected, directly or indirectly with the metabolic processes more intimately concerned in the laying down of collagen." The fact that at different levels of vitamin C-deficiency there is such a close correlation between the degree of differentiation of the scar tissue and its phosphatase activity is consistent with such a view. In this connection it is interesting that Wolbach and Bessey tentatively suggest that the mechanism by which ascorbic acid promotes the formation of collagenous intercellular substances " may be involved in the chemical mechanisms (enzymes) of the cells responsible for the synthesis of this protein product (collagen)."

Our results on the scorbutic animals, however, cannot be regarded as direct proof of a connection between alkaline phosphatase and the formation of collagen. It is conceivable that there may be no causal relationship between the absence of the enzyme and the poor differentiation of the collagen fibres, but that both phenomena may be due to some unknown third factor. For example, it is possible that the phosphatase in the wound may be supplied at an undiminished rate, but that for some reason it is not retained by the abnormal scorbutic scar tissue. A similar suggestion has been made by Bourne (1945) in connection with the healing of bone wounds in scurvy.

Alternatively there may be less phosphatase available in the scar, either because the cells of the regenerating tissue are producing less or, if the enzyme is derived from the blood stream, because the blood-phosphatase is lowered in scurvy (Shwachman and Gould, 1942*a*, *b*, and 1943), and the scars are poorly vascularized. Finally, it is not yet known whether this association between phosphatase activity and the regeneration of fibrous tissue exists in species other than rodents.

SUMMARY.

1. The effect of different degrees of vitamin C-deficiency has been studied on the rate of healing, phosphatase activity and differentiation of the scar tissue in small open skin wounds in guinea-pigs.

2. The rate of healing, epithelialization and contraction of the wound were greatly retarded in animals receiving respectively 0·5, 0·7 or 1·0 mg. ascorbic acid daily. Severe adhesions to the base of the wound in these groups were noted. At the 2 mg. level healing was more normal, and phosphatase activity, though subnormal, was increased.

3. The optimum level of vitamin C as assessed by its action on rate of healing, differentiation and phosphatase activity, is not less than 5 mg. of ascorbic acid a day, and possibly even higher.

The authors are indebted to the Medical Research Council, by whom the expenses of the investigation were in part defrayed, and to Dr. A. Glücksmann for valuable suggestions and advice. They also wish to thank Mrs. Danielli and Mr. G. Winter, assistant at the Strangeways Research Laboratory, for their help with the histological technique, and Mr. S. Impey, assistant at the Dunn Nutritional Laboratory, for his care of the animals. The photomicrographs were taken by Mr. V. C. Norfield, Senior Assistant at the Strangeways Research Laboratory.

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DIFFERENTIAL CENTRIFUGALIZATION OF INTRANUCLEAR PARTICLES IN THE BEAN ROOT AND IN ANIMAL TISSUES.

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Received for publication October 15, 1945.

BEAMS and King (1936) and other workers have shown that the nuclear contents of most cells are readily stratified into layers by centrifuging at high speeds, so that the nucleolus is displaced to the centrifugal pole, the basichromatin and oxychromatin are next in order, and the nucleoplasm is concentrated at the centripetal pole. We now show that when the resting nucleus is subjected to a centrifugal force of 60,000 times gravity, the extranucleolar chromatic particles composed of desoxyribonucleic acid protein are readily sedimented, whereas those composed of ribonucleic acid protein show little or no sedimentation.

EXPERIMENTAL.

The tissues were spun in the air-driven spinning-top type of ultracentrifuge described by McIntosh (1935) and McIntosh and Selbie (1937). Animal tissues were placed in the bottom of the centrifuge tubes, which were then filled up with Tyrode solution. Bean roots, grown in water to a length of 10 cm. and cut off 1 cm. from the tip, were supported and oriented by embedding tip downwards in 25 per cent gelatin in the centrifuge tubes. The tissues were then spun at 45,000 r.p.m., giving a centrifugal force gradient of 57,000 to 63,000 times gravity for the 3 mm. of tissue farthest from the centre of rotation. The tissues were spun for various times, but the structural changes to be described here seemed to be already complete after spinning bean roots for 30 minutes and animal tissues for 60 minutes.

The changes observed in the nuclei of bean roots are shown in Fig. 1 and 1*a*, where it can be seen that the nuclear wall is pulled out to an hour-glass shape, with the nucleolus and much of the chromatic material sedimented to the centrifugal pole and with unsedimented chromatic particles in the centripetal pole, some of which appear to have adhered to the nuclear wall and become elongated during spinning, thus giving the appearance of having been vertically stretched. Evidence of the nature of the two types of extranucleolar particle has been obtained by staining with Feulgen's method and by the use of ribonuclease. In Fig. 2 and 2*a* it is clearly shown that only the Feulgen-stained particles have been sedimented along with the nucleolus, while in sections that have been treated with ribonuclease the unsedimented particles have been digested, so that subsequent non-specific staining with Unna's stain or iodine gentian violet fails to stain these particles and gives the general appearance of a Feulgen-stained preparation. It would thus appear that the unsedimented particles belong to the class containing ribonucleic acid.

Similar, though less striking, observations have been made on nuclei of normal mouse liver and kidney, a transplantable rat fibro-adenoma (Selbie, 1942), and the Rous fowl sarcoma. In sections of spun specimens of these tissues stained with

haematoxylin and eosin the deeply basophilic chromatic particles are sedimented, whereas there is little or no sedimentation of the less basophilic ribonucleic acid particles, many of which can be seen to be vertically oriented and adherent to the nuclear wall (Fig. 3). With Feulgen staining it can be seen, as in the bean roots, that only the sedimented particles are stained (Fig. 4).

DISCUSSION.

These observations clearly show that the ribonucleic acid type of extranucleolar particle behaves differently from the desoxyribonucleic acid type under high-speed centrifugalization. In a more simple system, where the particles are suspended freely in a viscous medium, the main factors which would contribute to the more rapid sedimentation of one type of particle would be higher specific gravity, greater size, and more compact shape of the particle. It would appear improbable that a difference in the specific gravity of the two types of nucleoprotein would have any appreciable effect on their relative sedimentation velocities, but it is possible that the desoxyribonucleic acid particles are of more solid structure and thus have a greater specific gravity than the ribonucleic acid particles, which, judging from our observations, may be nearly equal to the nucleoplasm in specific gravity. The effect of the size of the particle is probably the main determining factor in the behaviour of the nucleolus, which is rapidly sedimented, although it is composed of the same type of nucleoprotein as the unsedimented particles. The size factor would also appear to contribute to the difference in the behaviour of the extranucleolar particles, because those of the desoxyribonucleic acid type appear somewhat larger on microscopical examination. The shape of the particle may also be of some importance in that the ribonucleic acid type may have a less regular surface, and thus offer more resistance to sedimentation in the viscous nucleoplasm than the desoxyribonucleic acid particles.

There are, however, certain features of the complex structure of the nucleus which may have a preponderating effect on the sedimentation of the nuclear contents. One important feature, which we have already mentioned, is the attachment of ribonucleic acid particles to the nuclear membrane which would effectually prevent sedimentation of these particles. The desoxyribonucleic acid particles, on the other hand, are attached, not to the nuclear wall, but to the linin network along with the nucleolus, so that these structures are relatively free from external support and thus can be more readily sedimented. It may therefore be concluded that the difference in the sedimentation behaviour of the two types of nucleoprotein particle is determined by a number of factors, of which the relationships of the particles to other structures in the nucleus may be of considerable importance.

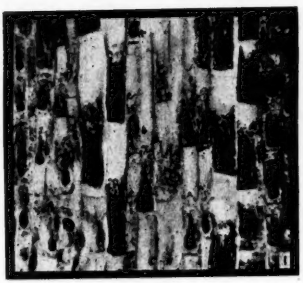
FIG. 1.—Bean root, spun at 45,000 r.p.m. for 30 minutes. The differential sedimentation of the nucleic acid protein particles is best shown in the central elongated cells. An explanatory drawing of the cell towards the left of the figure is shown in Fig. 1*a*. Carnoy fixation; Heidenhain's iron haematoxylin. × 240.

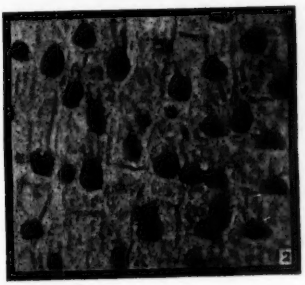
In all the figures the direction of the centrifugal force is towards the bottom of the figure.

FIG. 2.—Bean root, spun at 45,000 r.p.m. for 30 minutes, fixed in Carnoy's fluid and stained by Feulgen's method. The sedimented extranucleolar particles are stained while the unsedimented particles are unstained. × 350. An explanatory drawing of a representative cell is shown in Fig. 2*a*.

FIG. 3.—Transplantable rat fibro-adenoma, 8D, No. 3L, biopsy at 10 months, spun at 45,000 r.p.m. for 60 minutes. Most of the chromatic material in the nuclei is sedimented, while the upper parts of the nuclei contain more faintly staining strands with vertical orientation. Zenker fixation; haematoxylin and eosin. × 480.

FIG. 4.—As in Fig. 3, but stained by Feulgen's method to show that only the sedimented particles are stained. × 480.





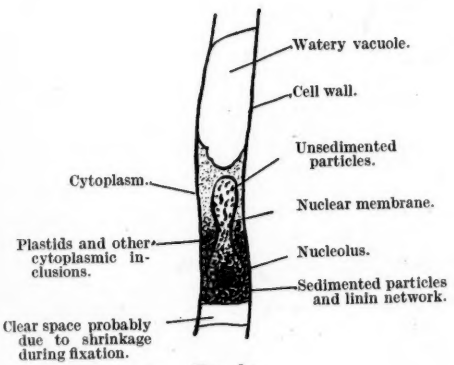

Watery vacuole.
Cell wall.
Unsedimented particles.
Cytoplasm.
Nuclear membrane.
Plastids and other cytoplasmic inclusions.
Nucleolus.
Sedimented particles and linin network.
Clear space probably due to shrinkage during fixation.

FIG. 1*a*.

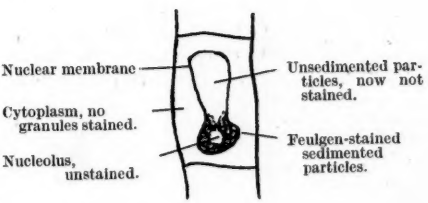
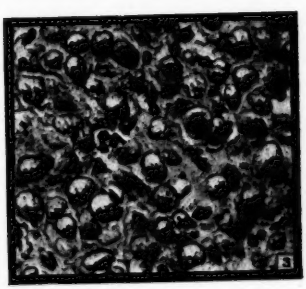
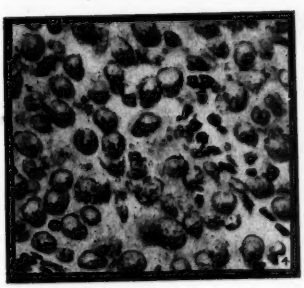

Nuclear membrane
Unsedimented particles, now not stained.
Cytoplasm, no granules stained.
Feulgen-stained sedimented particles.
Nucleolus, unstained.

FIG. 2*a*.





SUMMARY.

It has been shown in bean roots and in animal tissues that there are intra-nuclear particles with the properties of ribonucleic acid protein that are not sedimented under a centrifugal force sufficient to sediment the nucleolus and the desoxyribonucleic acid protein particles.

We are indebted to Mrs. Holmes, of the Strangeways Laboratory, Cambridge, for the ribonuclease tests. For this purpose tissues were fixed in Sousa or Carnoy, serial paraffin sections were fixed on slides, one half of the sections of each slide was digested with ribonuclease, and then the whole slide of sections was stained.

The expenses of this research were defrayed by the British Empire Cancer Campaign.

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GREY LUNG VIRUS: AN AGENT PATHOGENIC FOR MICE AND OTHER RODENTS.

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Received for publication October 26, 1945.

WE describe in this paper a virus-disease which produces chronic lesions in the lungs of mice and other rodents. It possibly exists as a latent infection of laboratory mice and was brought to light by serial passage, but such has not been certainly established. The virus appears to be distinct from the mouse-pneumonia viruses described by Nigg (1942), Horsfall and Hahn (1940) and others.

Methods.

In the experiments to be described, intranasal passages of infective material (usually lung) were made by grinding with powdered Pyrex glass and Hartley's broth, containing 10 per cent horse serum, to make a 5 per cent suspension. Etherized mice were inoculated intranasally with 0·05 c.c. quantities, and other species received appropriate larger amounts. Dilutions of infective material are expressed throughout in terms of weight of fresh tissue.

Isolation of the Virus Strains.

Two strains of the virus have been found and studied; no differences have been found between the two. *Strain M* turned up at the Institute's Farm laboratories at Mill Hill. One of us inoculated six mice and two ferrets with an unfiltered suspension of the lung of a calf which had died of pneumonia two days after receiving an intra-

venous inoculation of B.C.G. vaccine. The acute pulmonary changes in the calf-lung were not those commonly accompanying an uncomplicated vaccine reaction, nor did they at all resemble those of the disease in mice now to be described; they consisted chiefly of polymorphonuclear exudate into the alveoli. The lungs were bacteriologically sterile. One of the two inoculated ferrets, when it was killed 7 days later, showed two small consolidated areas at the hilus of the left lung, but nothing was transmissible on passage. The other ferret did not apparently react at all. The mice, killed 14 days after inoculation, showed no macroscopic lesions; a suspension of their lungs was passed to six more mice, and the latter, when killed 9 days later, showed diffuse grey areas in their lungs. After one more passage extensive lung consolidation was produced.

Strain E had its apparent origin in a filtrate from the lung of a fatal case of infantile diarrhoea in a girl 2 months old. This was inoculated intranasally into two etherized ferrets. One remained normal; the other was killed after 7 days and, though it showed only slight lung changes of doubtful significance, passage from a suspension of this lung was made to another ferret. This ferret reacted with fever of 105° F. on the 6th day, and, when killed, showed extensive red mottling of the lungs, due to acute localized broncho-pneumonia. Some of this lung, together with the congested nasal mucosa and turbinate bones, was sent to the Hampstead laboratory, where unfiltered suspensions of lungs and turbinates respectively were inoculated into groups of 9 mice. Intranasal passages from their lungs were made after 3 days, though no lesions were seen. The lungs of the mice of the "lung" and "turbinate" series were then combined and passed again intranasally. Seven days later mice of this third lot were killed and three of the six showed areas of greyish-red consolidation. The lesions proved to be transmissible in series indefinitely, but no really extensive ones were seen until after 7 passages.

Symptoms and Course of Disease.

After a series of passages, 10^{-6} dilutions of infective material could produce changes involving the larger part of the lungs of intranasally inoculated mice. Such mice rarely showed pronounced symptoms; their fur was perhaps rather rough and their gain in weight less than that of controls, but few died of the disease. More remarkable, the mice receiving undiluted virus, representing one million or more minimal infective doses, showed similar lack of definite symptoms and likewise few died. As will be shown, the lung lesions had little tendency to regress, nor did the virus tend to die out; the infected mice were, however, surprisingly well able to carry on with the lung tissue left to them, and even to rear litters.

Morbid Anatomy.

Mice killed within *3 or 4 days* of inoculation rarely showed more than isolated greyish-red consolidated areas on the lungs. After *6 to 8 days* lesions were much more extensive, often extending over a large part of the lung-surface; but after *14 days* an even better and very characteristic picture had developed. Lungs were voluminous, with no tendency to collapse when the chest wall was opened. 16 days after infection they were over twice as heavy (2·4 g : 1·1 g.) as lungs of comparable normal mice, and 50 per cent heavier than those of mice with complete consolidation due to influenza virus. They had a wet, reddish-grey appearance, looking not nearly as purple as do lungs infected with the viruses of influenza or mouse-pneumonitis (Nigg, 1942). Some lobes were almost wholly consolidated, but a lens usually revealed some small areas of emphysema on the lung-surface. Other lobes might show only extensive

mottling. Lungs of mice receiving limiting dilutions at times looked normal at first glance, but the lens revealed scattered small grey spots. We failed, however, to use the counting of these spots as a basis for estimation of virus titre ; too often the lesions were diffuse from the outset. On being cut into, the lungs of heavily infected mice exuded much clear watery fluid ; the wet appearance was clearly due to extensive oedema. A Petri dish into which six or more lungs had been collected often yielded 2 or 3 c.c. of this fluid. If mice bearing extensive lesions were etherized early in the disease there was an enormous outpouring of fluid from the nose ; this proved to be infective. In mice killed at any later time up to six months from inoculation the lesions did not appear to have regressed. The moist oedematous appearance became less and emphysematous areas were more evident, but lungs were still voluminous and not apt to collapse, and there was no tendency for them to become smaller from fibrosis, as happens within a few weeks in influenza-infected mouse lungs.

Morbid Histology.

The histological changes produced by the virus are of a varied and remarkable character ; it is intended to describe them in detail in a later paper. Most conspicuous are the lesions of interstitial pneumonia, and the collections of rather large mononuclear cells which form thick cuffs around the blood vessels and bronchioles. Later, scattered nodules of similar tissue are found, not in obvious relation to vessels. Other features of the early stages are the diffuse oedema which one would expect from the naked-eye appearances ; and obliteration of alveoli, without collapse, associated with swelling and proliferation of cells in the alveolar walls. In mice killed after several months the cuffs may be much as before, or the proliferating tissue may have extended to occupy as much as half the lung-volume. The alveolar cells may have enlarged still further, giving the appearance of a giant-cell pneumonia, or they may be undergoing foamy degeneration.

Emphysema and bronchiectasis may be conspicuous at later stages, and secondary bacterial infection may then occur, causing late deaths ; it is uncertain whether bronchiectasis follows infection or vice versa. As striking as the positive findings is the absence of evidence of fibrotic contraction of the lung as a whole. The bronchial epithelium is at first unaffected, but later the cells from the cuffs sometimes seem to press in and replace it. Lesions tend to be static or slowly progressive for many months ; the end-results of the processes are not yet known.

Haematology.

Blood counts on infected mice between the 11th and 21st days after infection showed a leucopenia (e.g. a fall from 12,000 to 4400 W.B.C. per c.mm.) and an appreciable increase in erythrocytes (e.g. from 9 to 12×10^6 per c.mm.). In view of the extensive pulmonary oedema, which is a marked feature of the disease and is well established by the 12th day, it is not unlikely that the high red cell count is an indication of anhydraemia.

Infectivity for the Mouse.

The agent is readily transmitted by the intranasal instillation of suspensions of mouse lung. Serial transferences are best made at intervals of not less than six to seven days, preferably after 10 days or even longer. After the administration of low dilutions (10^{-2}) virus can be detected at the 3rd day, although no visible lesions can be seen, but further passages at intervals of 3 to 4 days result in the extinction of the virus

by the 3rd passage. The failure of the virus to establish itself when passes are made at short intervals is probably due to the relatively long incubation period.

Titration experiments on mouse lungs at different intervals after infection showed that at the fourth day virus was present at a dilution of 10^{-1}, but not at 10^{-2}; at the 7th day extensive lesions were produced by dilutions of 10^{-3} and a minimal reaction by 10^{-4}; dilutions of 10^{-5} and 10^{-6} did not, at that stage, evoke recognizable lesions, but virus was detected by further passages in mice. From the 10th day onwards the end-point in respect of lung lesions and the presence of virus usually lay between 10^{-6} and 10^{-7}. It must be pointed out, however, that the assessment of the minimal infective dose by the visual changes in the lungs is not easy; indeed, we are not yet decided on the most favourable period after infection at which to kill the mice in order to secure a reasonably sharp end-point. Throughout the experiments mice used in titration trials have been killed, arbitrarily, about the 15th day, but further experience may show that this is not the optimum time.

A remarkable feature of the disease is the persistence of virus in the lungs for many months; indeed, an end-point has not yet been reached. At the 69th day after infection the lungs showed a diffuse greyish mottling, and a dilution of 10^{-8} produced characteristic lesions in fresh mice, whilst after $7\frac{1}{2}$ months, when lung lesions were as evident as before, a 10^{-5} dilution was positive.

Routes of Infection.

The virus is strictly pneumotropic. In spite of the extensive lung lesions which follow intranasal inoculation, spread of infection to other tissues does not occur at any stage. In one experiment, mice infected with a 10^{-2} dilution were killed at intervals of three days between the 4th and 27th day after infection. Blood taken from the abdominal vessels and suspensions of liver, spleen and kidney were transferred intranasally to fresh mice. The results were completely negative. The virus does not easily establish itself even in the snout.

Attempts to set up infection by routes other than intranasal instillation have been relatively unsuccessful. Large doses given subcutaneously fail to produce visible changes; nor can the virus be recovered from the spleen or lungs. In one of two experiments virus was recovered from the lungs after heavy intraperitoneal inoculation (22 days later). Mice inoculated intracerebrally remain normal, and no evidence of the establishment of the virus in the neural tissues was obtained by serial intracerebral passes or by the intranasal instillation of suspensions of brain. Lung lesions were, however, produced by intravenous injection.

Contact Infection.

Ten experiments were carried out in which normal mice were allowed to run in the same cage as infected animals. In experiments in which the periods of contact were 20, 20, 56, 90 and 90 days respectively, contact infection took place. This was shown by the occurrence of grey lesions of varying extent in the lungs of one or more mice, and the production of typical lesions on intranasal passage from their lungs to further mice. As a rule (3 of 4 trials) there was only evidence that 1 out of 4 contacts was infected. In other experiments (periods of contact 15, 18, 20 and 30 days) no contact infection as judged by these criteria occurred. In one trial with 18-day contact, the fresh mice developed no lesions, but virus was recovered from them after two serial passages. It seems, then, that contact infection occurs irregularly, and is more likely to occur if mice are together for many weeks. In harmony with this result are experiments on intranasal infection without the use of anaesthetics. No lesions were found

in the lungs of two mice killed 11 days after such inoculation, though virus was present ; 6 months after inoculation, without anaesthetic, each of three infected mice showed typical lesions and virus was recovered on passage ; a similar result was obtained after 9 months. No virus was recovered from lungs or snouts 2, 3 or 4 days after inoculation of mice without anaesthetic.

Physical Properties.

Infective lung taken up in serum broth retains its potency when conserved in CO_2-alcohol (— 76° C.). No appreciable loss in infectivity has been detected after 5 months (not an end-point). At room temperature the titre was unchanged after 6 hours ; in this respect the virus differs from that of Horsfall and Hahn (1940), which is so sensitive that it becomes completely inert within 2 hours at room temperature. At 37° C. a filtrate was completely inactivated overnight. The virus was destroyed by immersion in a water-bath at 50° C. for half an hour. Complete inactivation of unfiltered virus was also effected by the action of 0·3 per cent formaldehyde at cold room temperature (24 hours), and 0·02 per cent formaldehyde inactivated a filtrate after 4 days in the cold. It has been possible to preserve the virus by drying from the frozen state.

It is not proposed in this paper to give full details of the physical properties of the virus, since these will be reported in a further communication. The agent is filtrable through " gradocol " membranes of average pore diameter of 450mμ. and upwards ; it is also sedimented by high-speed centrifugation. Filtered virus is stable at room temperature for at least 24 hours, when suspended in 10 per cent serum broth.

Transmission to Other Species.

In addition to the mouse, the virus has been transmitted to a variety of rodents, but except in the cotton-rat, it tends to produce mild lesions or may persist in a latent form.

In rats, hamsters (*Cricetus auratus*) and voles (*Microtus arvensis*), conspicuous pulmonary lesions were but rarely encountered ; nevertheless, virus was at times present in a relatively high concentration, e.g. in the rat virus was detected in dilutions of 10^{-5} at the 14th day, 10^{-4} at the 21st day, 10^{-5} at the 46th and 61st days after infection. Serial passes through rats at intervals of 7 to 10 days failed to enhance the virulence, and lesions at the 20th passage were no more extensive than those induced initially in spite of a persistently high titre. Similar results were obtained in the hamster ; virus-titres in the lungs were 10^{-5} at the 46th and higher than 10^{-5} at the 61st day. Results in the vole were more irregular. In one experiment virus was recovered from one vole out of five (after 19 days) ; in another, virus was recovered after 10, 18, 25 and 40, but not 7 days ; in several other experiments no virus was recovered ; virus titres were always low.

In the guinea-pig the virus produced only trivial lesions, but virus was detected by a single mouse passage after 13 days.

No definite macroscopic or microscopic lesions were produced by intranasal inoculation into rabbits. Virus was, however, recovered from the lungs of rabbits killed after 17 and 37, though not after 87, days. At 37 days only minimal quantities of virus were obtained, a 1 : 1000 dilution giving negative results on inoculation into mice, and virus did not survive passage through a second rabbit.

In cotton-rats the grey lung virus produced scattered or diffuse grey lesions resembling those in the mouse. The perivascular cuffing and swelling of alveolar cells, also, resembled the mouse's reaction. Stronger concentrations of virus, however, were

more lethal in cotton-rats than in mice ; even a 1 : 1000 dilution killed 3 of 6 animals (after 7, 11 and 13 days). Such cotton-rats showed acute lung consolidation with oedema and exudate of polymorphonuclear cells. Though the lethal action was not constant, we were satisfied that these deaths were due to the virus itself and not to any associated bacteria. In cotton-rats which survived, the infection followed a chronic course and virus persisted as in mice ; lung suspensions titrated in mice were infective to a dilution of 10^{-5} after 2 months and the same after 3 months.

It was mentioned in the early part of the paper that one of the strains isolated was apparently derived from material passed through ferrets. We were surprised to find that subsequent attempts to establish the virus in this species by means of mouse passage material were unsuccessful. Thus two ferrets inoculated with a 10^{-2} dilution of infective mouse lung (approximately 10,000 m.i.d.) in the 21st generation in mice were free from lesions when killed at the 8th and 28th days respectively ; suspensions of their lungs failed to infect fresh mice. It is conceivable that initially the virus was feebly pathogenic for ferrets, but as a result of adaptation to mice lost its capacity to infect ferrets, or susceptibility on the part of the ferret may be an individual idiosyncrasy. A third possibility is that the ferret material stimulated a latent infection in the normal mouse stock.

Mouse virus given intranasally under anaesthesia to a 4-months-old lamb failed to evoke recognizable lung lesions and virus was not recovered after 20 days.

Attempts to establish the agents in developing eggs by dropping filtrate of mouse lungs on to the chorio-allantoic membrane, or by inoculating the allantoic and amniotic cavity and yolk sac, have been fruitless. Attempts at tissue culture with minced chick embryos in Tyrode solution were also negative.

Immunity.

So far it has not proved possible to demonstrate an active immunity in mice. On three occasions groups of mice were inoculated intraperitoneally at weekly intervals with a concentrated suspension of infective lung (two or three doses). Three weeks later the inoculated mice together with controls of a similar weight were divided into groups which received falling doses of living virus intranasally. No significant difference in the extent of the lesions was detected as between vaccinated and control groups.

A similar experiment in which we used virus inactivated with 0·02 per cent formaldehyde was equally fruitless. Neutralizing antibodies have not been found in the serum of mice inoculated intraperitoneally with living virus or intranasally with small doses of virus. Serum from rats inoculated intranasally 28 days previously with mouse lung also failed to neutralize the virus. Other serological studies (e.g. complement-fixation tests) are in progress, but no positive results have yet been obtained.

Differentiation of the Virus.

(a) *From the mouse-pneumonitis virus* (Nigg).

The stocks of mice with which we have been working have unfortunately not been free from mouse-pneumonitis virus. On several occasions we have isolated from our normal mice an agent corresponding with that described by Nigg (1942). This virus is probably identical with that described earlier by Dochez, Mills and Mulliken (1937), and by Gordon, Freeman and Clampit (1939). Our strain has readily attained a high virulence, killing mice with lung consolidation in a few days when inoculated intranasally, but not lethal when inoculated intracerebrally or intraperitoneally. Elementary bodies are readily found, chiefly in polymorphonuclear cells, in lung smears

stained by Machiavello's method ; and bodies can be found in mononuclear cells in lung sections as described by Furth and de Gara (1944). The agent is susceptible to chemotherapy with sulphadiazine or sulphathiazole. At least twice during passages of grey lung virus, the mouse pneumonitis virus has apparently been picked up and has begun killing mice within a day or two. Its presence has been recognized by this lethal action and the presence of the characteristic elementary bodies. We have always been able to obtain the grey lung virus pure once more by going back to stored material or to mice inoculated before the pneumonitis virus appeared. The grey lung virus is distinguished from it by its smaller size, as shown by passage through a 0·4 μ membrane, by its being completely unaffected by sulphonamide drugs, and by not giving rise to the characteristic elementary bodies. We have found, in some smears of " grey lungs " stained by Machiavello, red-staining bodies definitely smaller in size than those of the pneumonitis virus and appearing where a mononuclear cell has been ruptured, and not contained, as with pneumonitis virus, within polymorphonuclears.

(b) *From the pneumonia virus of mice (PVM) of Horsfall and Hahn* (1940).

We have not had the opportunity of direct comparison of our agent with the PVM virus. Ours differs from it in not killing mice at all readily, in not being readily labile in suspensions held at room temperature, in not being inactivated by a specific PVM serum, kindly sent us by Dr. F. L. Horsfall, and in not agglutinating mouse red cells (*cf.* Mills and Dochez, 1945).

(c) *From the virus of lymphocytic choriomeningitis.*

The grey lung virus is not pathogenic for mice when inoculated intracerebrally. Filtration indicates that it is considerably larger than the lymphocytic choriomeningitis virus. To make further certain of its non-identity we challenged four mice which had been inoculated with grey lung virus (first intraperitoneally, three times, and then intranasally) by intracerebral inoculation of choriomeningitis virus ; they died *pari passu* with controls.

(d) *From pleuropneumonia-like organisms.*

From lungs, both of normal mice and those infected with grey lung virus, we have frequently cultivated pleuropneumonia-like organisms in 10 per cent horse-serum broth, but we have often failed to grow anything from grey lung mice in broth or on agar containing 10 or 30 per cent horse-serum or in other media. The organisms recovered have at times corresponded to Kleineberger's " L 5," which produces rolling disease in mice ; at other times they have been of a type, to be described elsewhere, showing definite motility. Cultures of the latter organism, which may be identical with one of those described by Edward (1940), produce lung lesions on intranasal inoculation into mice and, histologically, dense cuffs round vessels not unlike those of grey lung. The lesions are, however, commonly circumscribed ; one does not find the voluminous oedematous lungs characteristic of the early stages of grey lung ; and the lesions produced by the pleuropneumonia-like organisms have not been regularly reproducible in series. We cannot exclude the possibility that the lesions of grey lung are caused by one agent of this class which has as yet eluded our attempts to cultivate it.

We feel fairly confident that the agent now described differs from any of those above mentioned. Nor does it seem to correspond with any of the " mouse pneumonia viruses " recently described from Germany. That of Gönnert (1941) seems

likely to be the same as Nigg's. Those recorded by Herzberg (1943) and Gross (1943) comprise a pleuropneumonia-like organism, a virus which kills mice in two or three days and a mixture of the two agents. Heinzmann's (1941) agent was not filterable, and caused chronic lung lesions beginning as purulent bronchitis. Nelson's (1937) coccobacillary bodies were not found, nor do the clinical and pathological features of "grey lung" correspond with what he describes.

The long-term course and the macroscopic appearances of the "grey lung" are, with well-established virus, characteristic. Unfortunately we have not yet found any characteristic inclusion-body, unique histological feature nor specific immunological test enabling us to recognize with certainty the presence of small quantities or possible milder strains of the virus. The dense perivascular cuffs of rather large mononuclear cells are a striking and regular feature of its activities in the lung, but they are not specific, and may be a part of the reaction to a number of different agents.

Origin of the Virus.

Four attempts have been made to isolate fresh strains of virus by serial passage in mice, using ground-up lung and inoculating intranasally under ether. In three series we began with the inoculation of normal mouse lungs; two of these series were carried on for 8 passages, first at intervals of 9–10 days between passages, later at fortnightly intervals; a third series was lost after 6 passages through occurrence of bacterial infection. In a fourth attempt the lungs of the first mouse in the series were damaged by intranasal inoculation of a weak diphtheria toxin; then three serial passages were carried out at fortnightly intervals. In all the series perivascular cuffs were seen histologically in the lungs, but no macroscopic lesions were produced except in the presence of demonstrable infection with another agent.

Further, one of us did not bring to light the grey lung virus in the course of 18 months' work in 1943 and 1944 on the etiology of human atypical pneumonia; in the course of this many lots of material were passed through mouse lungs at 7- to 10-day intervals. Despite these negative results, activation of a latent virus in our mice seems a more likely origin for the virus than other possibilities which have occurred to us. Our two strains may be highly active variants of an agent which is commonly present but difficult to detect.

SUMMARY.

The two authors of this paper each isolated in the summer of 1944 a mouse-pathogenic virus, one starting with material from a calf-pneumonia, the other with some from infantile diarrhoea. The two strains seem to be identical, and to differ from any virus hitherto described. This "grey lung" virus may have originated in the normal mouse stock, but subsequent attempts to obtain strains from the normal mice have failed. The virus is strictly pneumotropic, but commonly not fatal, though infective in high dilutions. It produces in the lungs chronic lesions which remain relatively stationary for many months. Suspensions of lungs are infective in high dilutions for at least 9 months—the longest period yet tested—after infection of the mice. The virus is contagious for mice running in the same cage as infected ones, but not very highly so. Neither active immunity nor production of antibodies has yet been demonstrated. The virus will produce chronic infection in cotton-rats and will infect other rodents, though it is less pathogenic for them. It is relatively stable, and will pass a gradocol membrane of A.P.D. 450 mμ. Differences from other mouse-pathogenic agents are discussed.

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A SPECIFIC BLOOD GROUP O SUBSTANCE.

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Received for publication October 29, 1945.

THE original classification of human bloods into four groups, A, B, AB and O, was based on the presence or absence of the agglutinable substances A and B within the erythrocyte. The occurrence or absence of the specific group factors A and B in the red cells was determined by means of agglutination tests employing the agglutinins anti-A and anti-B which occur naturally in the serum of group B and A persons respectively. For many years group O erythrocytes were considered to be cells devoid of the agglutinogens A and B, and were recognized by the absence of these biologically important factors, and not by the possession of a specific and characteristic agglutinogen of their own. According to Bernstein's theory, which is now generally accepted, erythrocytes of the genotype OO possess a specific O factor corresponding to the A and B agglutinogens, but direct evidence for the existence of the O factor in erythrocytes was not obtained until a reliable and specific anti-O serum became available. Landsteiner and Witt (1926) and Landsteiner and Levine (1926) showed that occasionally the serum of persons belonging to the groups A_1 and A_1B contains an " irregular " isoagglutinin which reacts with O cells, and at about the same time Schiff (1927) and Greenfield (1928) reported that certain normal cattle sera, after absorption with A_1B erythrocytes, also agglutinated group O cells. It has generally been found, however, that the irregular iso-agglutinin and absorbed normal cattle sera are weak reagents which react with O erythrocytes at relatively low dilutions only, and are not to be compared in potency with the natural or immune anti-A and anti-B sera that are usually employed for the identification of blood groups. Hooker and Anderson (1921) produced an anti-O serum in a single rabbit by immunization with human erythrocytes belonging to group O, and suggested that group O cells contain a specific agglutinogen that does not exist in cells of the other groups. More recently Matta (1937) has confirmed the formation of anti-O sera in rabbits

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by [the injection of group O erythrocytes. Eisler (1930, 1931, 1932*a* and *b*) succeeded in producing a powerful anti-O serum by the immunization of goats with *Bact. shigae*. The serum was thoroughly investigated by Landsteiner and Levine (1931), Schiff (1934) and by Hirszfeld and Amzel (1940) and found to be a very useful reagent. There appears to be no record of other workers producing a serum of this kind, and our attempts to induce the formation of an anti-O serum in three goats by immunization with a suspension of *Bact. shigae* or with the purified somatic antigenic complex of this organism (Morgan, 1937) were not successful. During the early part of the work described in this paper a naturally occurring anti-O serum from a cow was used, but later a more potent and equally specific anti-O serum was produced by immunizing rabbits with the O-substance that had been isolated during the investigation.

A material possessing group O activity has been obtained from group O blood by Hallauer (1934) and by Stepanov, Kuzin, Makajeva and Kosjakov (1940) and from gastric juice by Witebsky and Klendshoj (1941), but in each instance only small quantities of material were available for study, and the purity of the substance isolated was not established. Witebsky and Klendshoj obtained 185 mg. of serologically active material from 1 litre of pooled gastric juice obtained from secretors belonging to group O. The material is described as a white hygroscopic substance which as a 1 per cent solution fails to react with trichloracetic acid and salicylsulphonic acid in tests for protein. The Molisch test was strongly positive, the substance contained 2·8 per cent total nitrogen, and gave rise to nearly 40 per cent of reducing sugars after acid hydrolysis. Phosphorus was absent. Landsteiner and Harte (1941) have also described, in a preliminary communication, the isolation of a group O specific substance from human saliva. The yield of active substance was small, 500 ml. of saliva giving rise to about 15 mg. of the active material. The analytical figures for total nitrogen, amino-acid N, hexosamine N and reducing sugars as glucose, after acid hydrolysis fail to distinguish the O substance from the corresponding A and B substance isolated by a similar technique from human saliva.

Pseudo-mucinous ovarian cyst fluids obtained from women who secrete their specific blood group substance in a water-soluble form have been shown by Morgan and Van Heyningen (1944) to be a convenient and potent source of the group specific substances A, B and O. The isolation of the O substance from pseudomucinous ovarian cyst fluids is described in the present communication, together with an account of the chemical and immunological properties of the specific substance.

EXPERIMENTAL.

Anti-O agglutinin.

A natural anti-O serum selected for potency after the examination of over 100 normal cattle sera was used throughout the investigation as a " standard " agglutinin. One litre of the serum was collected and was kept at −20° without preservative. From time to time 30–50 ml. amounts of the undiluted serum were absorbed with fresh A_1B cells until the serum failed to react with the absorbing cells and with several specimens of A_1B cells. The absorbed serum was filtered through a sterile candle or Seitz filter, filled into 2–3 ml. ampoules, sealed and stored without preservative at − 20°. A fresh ampoule of serum was opened for each test, and before use the serum was tested with A_1B and O cells as a check on specificity and activity. The stability of the anti-O reagent was controlled further by carrying out at suitable intervals inhibition tests with an active O cyst fluid which had been dried *in vacuo* from the frozen state after clarification by thorough centrifugation in the closed bowl of a Sharples supercentrifuge. The reactions of the " standard " anti-O reagent are shown

in Table I. The serum gives rise to no significant agglutination with A_1B cells, whereas O cells are agglutinated at a dilution of 1 : 256. Any agglutination given by A_1 and B cells is generally considered to be due to the presence of an O component in the particular erythrocytes used, and that A_1 and B cells which react with anti-O agglutinin are really heterozygous and belong to the genotypes A_1O and BO. We have observed that certain erythrocytes belonging to group B react with anti-O serum as strongly as A_2 cells, whereas other group B erythrocytes give no more than a trace of agglutination when they are tested under the same conditions. This interesting behaviour of B cells is being investigated further, and will be discussed when a more detailed examination of a large number of group A and B erythrocytes has been undertaken with a rabbit anti-O agglutinin as well as with the usual cattle anti-O serum.

TABLE I.—*Showing the Agglutination of Erythrocytes of Different Phenotypes with Anti-O Cattle Serum.*

	Dilution of serum showing agglutination.						
Test cells.	1 : 4.	1 : 8.	1 : 16.	1 : 32.	1 : 64.	1 : 128.	1 : 256.
O	3	3	3	2	2	1	1
O	3	3	3	2	2	2	1
A_1B	0	0	0	0	0	0	0
A_1B	1	0	0	0	0	0	0
A_1	2	1	0	0	0	0	0
A_2	3	3	2	2	1	0	0
A_2B	1	0	0	0	0	0	0

Degrees of agglutination : 0, no agglutination ; 1, a few groups of 2–4 cells ; 2, larger groups with many free cells ; 3, small clumps visible to the naked eye ; 4, massive agglutination.

Inhibition and agglutination tests.

These tests were carried out as described earlier (Morgan and Van Heyningen, 1944).

The Isolation of the Specific O Substance.

Preliminary experiments, made on material obtained from cysts of low activity, showed that methods similar to those already developed for the isolation of A substance (Morgan and King, 1943) could be employed for the isolation of the O substance.

Ten g. of the crude O substance (total weight 31 g.) obtained by drying a suitable cyst fluid was extracted with 90 per cent liquid phenol at room temperature. A considerable part of the material dissolved, including most of the yellow pigment, and left behind a colourless, gelatinous substance. A second and third extraction of the insoluble material with 90 per cent phenol was carried out to remove the last traces of the soluble protein components. The residue was then repeatedly treated with ethanol to remove phenol, and finally dissolved in water, dialysed and dried from the frozen state. The combined phenol extracts were treated with ethanol with thorough shaking until, at a concentration of 30 per cent ethanol, a precipitate was formed. The substance thrown out of solution between 30 per cent. and 50 per cent ethanol was collected by centrifugation, washed with ethanol and dried.

The material insoluble in 90 per cent phenol contained 7·3 per cent N, showed a specific rotation $[\alpha]_{5461} - 32° \pm 2°$, and inhibited the action of 3–4 completely agglutinating doses of anti-O serum at a dilution of $1 : 64 \times 10^3$. The material precipitated from phenol solution by ethanol between 30 and 50 per cent concentration showed no considerable capacity to neutralize anti-O agglutinin and was not investigated further. The active, phenol insoluble material was dissolved in water at 0° by the

addition of the minimum amount of alkali and immediately neutralized. The solution was then warmed to 37°, and powdered Na_2SO_4 was slowly added to the thoroughly stirred solution. At a concentration of 26 per cent Na_2SO_4 a precipitate began to form, and the material separating between 26 per cent and 33 per cent Na_2SO_4 was collected by centrifugation, dissolved in water, dialysed at 0° until free from Na_2SO_4 and dried *in vacuo* from the frozen state. The material contained 5·7 per cent N, possessed a *laevo* rotation, $[\alpha]_{5461} - 23° \pm 3°$, and showed considerable viscosity, η 2·18 (0·5 per cent in 0·9 per cent saline). The specific serological activity of the material had increased, and 1 : 128 × 10^3 dilution of the preparation completely inhibited the agglutinating action of the " standard " anti-O serum on O cells. Electrophoretic examination of the O substance at pH 8·0, however, showed the presence of two components, one of which occurred in small amount only and migrated at pH 8·0 with almost the same speed as the major component. The Na_2SO_4 precipitation process was repeated on the active material, and a fraction separating between the levels 25 per cent and 30 per cent Na_2SO_4 was collected, dialysed and dried. The material (1·7 g.) contained 5·7 per cent N, possessed a specific rotation $[\alpha]_{5461} - 22° \pm 2°$, but again showed a small but reduced amount of a second component when examined in the electrophoresis apparatus. A fraction separating just below 25 per cent Na_2SO_4 concentration was found to possess a slightly lower specific activity than the main fraction, but to show a very similar specific rotation and N content.

The Chemical and Immunological Properties of the O Substance.

The material, obtained by drying *in vacuo* from the frozen state a neutral aqueous solution of the O substance, is of a white silky appearance. One preparation gave the following analytical figures: C, 44·5; H, 6·6; N (Dumas), 5·9; ash, 3·3 per cent. The substance, which is somewhat hydroscopic and will take up 10–15 per cent of moisture if left in the air, dissolves in water or saline, to yield a 1 per cent solution which shows considerable viscosity. The aqueous solution does not precipitate when treated with an equal volume of 2 per cent solutions of the following reagents: picric acid, aluminium sulphate, phosphotungstic acid, lead acetate, ferric chloride, potash alum and silver nitrate. Fehling's solution fails to precipitate the O substance from aqueous solution. The O substance gives no positive colour test when treated directly with *p*-dimethylaminobenzaldehyde reagent, but gives rise to a strong purple colour if previously heated at 100° for a few minutes with 0·05 N Na_2CO_3 according to the method described by Morgan and Elson (1934). The maximum colour developed under these conditions is equivalent to about 18 per cent of N-acetylglucosamine. At the same time the O substance develops reducing properties equivalent to about 4–5 per cent glucose as determined by the copper reduction method of Somogyi (1937). Hydrolysis of the O substance with 0·5 N HCl at 100° gives rise to hexosamine equivalent to 33 per cent of glucosamine and to 48 per cent of reducing sugars expressed as glucose. Hydrolysis with 5N HCl reveals that about 43 per cent of the total nitrogen is present as amino-acids, determined by the technique of Van Slyke, Dillon, McFadyen and Hamilton (1941). The liberation of α-amino groups during hydrolysis was followed by Van Slyke's method (1929), and showed that at least 81 per cent of the total nitrogen of the O substance is liberated in this form. A more detailed chemical examination of the O substance is deferred until material is available that is electrophoretically homogeneous when examined at pH 4·0 and 8·0.

The serological activity of the purified preparations of O substance was determined by means of the agglutination inhibition test, using 3–4 completely agglutinating doses of anti-O agglutinin. The results of the tests are given in Table II, where similar

TABLE II.—*Showing the Activity of Crude Cyst Fluids, Saliva and Purified O Substance in Agglutination Inhibition Tests with* (a) *Natural Anti-O Cattle Serum, and* (b) *Immune Anti-O Rabbit Serum.*

	Degree of agglutination with—															
	(*a*) Anti-O cattle serum.								(*b*) Anti-O rabbit serum.							
	Dilution of test substance (× 1000).*															
Test-substance.	1 : 2.	1 : 4.	1:8.	1:16.	1:32.	1:64.	1:128.	1:256.	1 : 2.	1 : 4.	1:8.	1:16.	1:32.	1:64.	1:128.	1:256
Crude O cyst substance (active)	0	0	0	0	0	0	1	2	0	0	0	0	0	0	1	2
Ditto	0	0	0	0	1	2	2	3	0	0	0	0	1	2	2	3
,,	0	0	0	1	2	2	3	3	0	0	0	1	2	3	3	3
Crude substance from inactive O cyst	2	3	3	3	3	3	3	3	2	3	3	3	3	3	3	3
Saliva No. 1	0	0	0	1	2	2	3	3	0	0	0	1	2	2	3	3
,, No. 2	0	0	0	1	2	2	3	3	0	0	1	2	2	3	3	3
Purified O substance	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	1

* Expressed on dry weight basis. Final dilution in test 3 times this figure. Degrees of agglutination as given in footnote to Table I.

results for a number of specimens of crude O substance and active saliva O-substance are included. The results show that the purified O-substance is considerably more active than the most active crude O material obtained by drying the native cyst fluid. The saliva specimens are the most potent we have encountered, but are nevertheless only as active as the weakest of the cyst fluids examined, and possess not more than 5 per cent of the activity of the purified O substance. It will be seen that the results obtained with cattle serum and an immune-rabbit anti-O agglutinin, the preparation of which is described later in this paper, are almost identical.

TABLE III.—*Showing the Agglutination of O Erythrocytes by Absorbed Anti-O Cattle Serum after Treatment of the Latter with A-Substance of Different Origin.*

	Degree of agglutination observed. Dilution of test substance (× 100).										
Test substance.	2·5.	5.	10.	20.	40.	80.	160.	320.	640.	1280.	2560.
Crude cyst A substance No. 54	0	1	2	3	3	3	3	3	3	3	3
,, ,, ,, No. 62	0	0	1	2	2	3	3	3	3	3	3
Purified cyst A substance M.9	0	1	1	2	3	3	3	3	3	3	3
,, hog mucin A substance	0	0	0	0	0	0	0	0	0	0	1
,, cyst O substance 61/11	0	0	0	0	0	0	0	0	0	0	1

Degrees of agglutination as given in footnote to Table I.

Sasaki (1932), Schiff and Sasaki (1932) and Witebsky and Klendshoj (1941) showed that saliva and gastric juice obtained from secretors belonging to group A and B inhibit the action of cattle anti-O agglutinin on human group O cells. Morgan and Van Heyningen (1944) largely confirmed these observations, but found that A and B substances present in a number of pseudomucinous ovarian cyst fluids failed to interfere with the agglutination of O cells by anti-O serum. A number of other active cyst fluids obtained from secretors belonging to group A have been tested since that time for their capacity to inhibit the action of O agglutinin on O cells, and the results of some of these tests, together with those obtained using purified and apparently homogeneous preparations of A-substance of both animal (hog) and human origin, are given in Table III. Tests, the results of which are not given in Table III, were made with an immune anti-O rabbit serum as agglutinating agent, as well as with the usual anti-O

cattle serum. The results show that the electrophoretically homogeneous A substance derived from hog gastric mucin inhibits strongly O agglutination, thus confirming the earlier observation of Morgan and Van Heyningen (1944). Indeed, the purified O substance itself is not noticeably more active in this respect than is this preparation of A substance. On the other hand, the electrophoretically homogeneous A substance isolated from ovarian cyst fluid does not inhibit, except at high concentrations, the action of anti-O agglutinin on O cells. A complete or partial inhibition of O agglutination by cyst A substance has been observed, however, when absorbed anti-O immune rabbit serum is used in place of the natural anti-O cattle serum. Crude cyst A substance (No. 62, Table III), which showed the most pronounced capacity of any cyst we have encountered to inhibit the agglutination of O cells by immune anti-O rabbit serum, inhibited at a dilution of 1 : 32,000, whereas the purified A substance (M9, Table III) inhibited O agglutination, under the same conditions, at a dilution of 1 : 2000 only. A number of non-specific substances, such as the undegraded specific polysaccharide components of the somatic antigens of *Bact. shigae* and *Bact. dysenteriae* (Flexner), (Morgan and Partridge, 1940, 1942), the Vi and O antigens of *Bact. typhosum* (Morgan and Partridge, 1942), undegraded dextran (Grönwall and Ingelman, 1944, 1945), hyaluronic acid (Meyer and Palmer, 1934), and kanten, the water-soluble hydrolysis product of agar (Takahashi and Shirahama, 1934; Pirie, 1936), were included in the inhibition tests. It may be said at once that these substances, with one exception, showed no significant inhibiting activity. The Vi antigen, however, at concentrations 1 : 250 to 1 : 2000, inhibited completely the action of cattle O agglutinin, but not of the immune anti-O agglutinin. It is of interest that the specific polysaccharide of *Bact. shigae* failed to cause any inhibition of O agglutination, especially in view of the observations of Eisler (1930, 1931, 1932*a* and *b*), Landsteiner and Levine (1931), Schiff (1934) and Hirszfeld and Amzel (1940), that an anti-*Bact. shigae* serum produced in a goat agglutinated O and A_2 erythrocytes.

Immunization Experiments.

A group of rabbits was given intravenously 6 doses, each of 0·1 mg., of the purified O substance and bled one week after the last dose. The sera were tested for their action on A, B and O cells, and were then absorbed, undiluted, with A_1B cells until they failed to react with the absorbing cells. The results of the agglutination tests are given in Table IV, from which it will be seen that anti-O agglutinins were produced

TABLE IV.—*Showing the O Agglutination Titres of the Sera of Rabbits Immunized with Six 0·1 mg. Doses of Purified O Substance after Absorption with A_1B Cells.*

Rabbit number.	Test cells.	Original O titre.	Dilution of serum giving agglutination.									
			1 : 2.	1 : 4.	1 : 8.	1 : 16.	1 : 32.	1 : 64.	1 : 128.	1 : 256.	1 : 512.	1 : 1024.
933	O	48	4	4	4	4	3	3	2	2	1	0
	A_1B	..	1	0	0	0	0	0	0	0	0	0
941	O	12	4	4	4	4	3	3	2–	2	1	0
	A_1B	..	?	0	0	0	0	0	0	0	0	0
942	O	<12	4	4	3	3	2	2	1	0	0	0
	A_1B	..	?	0	0	0	0	0	0	0	0	0
945	O	12	4	3	3	3	2	1	0	0	0	0
	A_1B	..	1	0	0	0	0	0	0	0	0	0
948	O	12	4	4	3	3	3	2	2	1	0	0
	A_1B	..	2	?	0	0	0	0	0	0	0	0
949	O	12	4	4	4	4	4	4	4	3	3	2
	A_1B	..	1	0	0	0	0	0	0	0	0	0

Degrees of agglutination as given in footnote to Table I.

by all the rabbits immunized. The serum from Rabbit 949 was very potent, and contained a much stronger anti-O agglutinin than any specimen of natural cattle serum we have examined. The average increase in the anti-A and anti-B agglutinin titres above the levels before immunization was six- and eightfold respectively. The corresponding increase in titre observed for the agglutinin reactive against O cells on the other hand was from an average value of 18 to 580 or about thirtyfold. The results indicate that the purified O substance is antigenic, and is capable of inducing the formation of quite potent agglutinins reactive against O cells. The response was largely specific, and there was no evidence that the material could elicit the formation of species specific antibodies as occurs when rabbits are immunized with O cells according to the technique of Hooker and Anderson (1921) and of Matta (1937).

Examination of Agglutinin Recovered from O Cells.

Anti-O immune rabbit serum (3 ml.) was absorbed with 1 ml. of washed group O cells. The cells were recovered after standing for 30 minutes at room temperature, washed three times with ice-cold saline, suspended in 2 ml. of saline and kept at 50–56° for a few minutes. The cells were then recovered by centrifugation at this temperature, and the clear supernatant fluid tested for agglutinins reactive with erythrocytes of different phenotypes. The results are set out in Table V, and show that the test cells react qualitatively with the recovered agglutinin in the same manner as they react with the unabsorbed reagent. Similar results were obtained with the agglutinin recovered from anti-O cattle serum. Experiments were also carried out using group A_2 cells in place of O cells. The agglutinin recovered from the cattle or rabbit anti-O serum by means of A_2 cells reacted with O, A_1, A_2, A_1B and A_2B cells in the same manner as did the agglutinin recovered from group O cells.

TABLE V.—*Showing the Results of Agglutination Tests Carried out with the Immune-Body Absorbed from an Immune Anti-O Rabbit Serum by O Cells and Subsequently Recovered.*

	Degree of agglutination. Dilution of recovered agglutinin.							
Test-cells.	1 : 2.	1 : 4.	1 : 8.	1 : 16.	1 : 32.	1 : 64.	1 : 128.	1 : 256.
O	4	4	3	3	3	3	2	1
A_1	0	0	0	0	0	0	0	0
A_2	4	3	3	2	2	1	0	0
A_1B	1	0	0	0	0	0	0	0
A_2B	0	0	0	0	0	0	0	0

Degrees of agglutination as given in footnote to Table I.

The Influence of Temperature on Group O Agglutination.

The degree of agglutination produced by natural and immune anti-O serum at different temperatures was investigated to determine whether the two reagents showed a difference in thermal amplitude. The O titre of the anti-O cattle serum at 6–8°, 18–20° and at 37° was 128, 32 and 2 respectively, whereas a specimen of anti-O immune-rabbit serum examined at the same time and under the same conditions showed agglutination endpoints of 256, 256 and 32. Both sera became somewhat unspecific, and reacted with A_1B erythrocytes at 6–8°.

DISCUSSION.

As a result of the earlier discovery that certain pseudomucinous ovarian cysts are a convenient and potent source of group specific substances A, B and O, it has been

possible to isolate an active group O substance from the fluid contents of pseudo-mucinous adenoma obtained from women belonging to group O who possess the power to secrete their blood group substance in a water-soluble form. A preliminary chemical examination of the O substance shows it to be very similar to the specific blood group A substance. The electrophoretic examination of the most active preparations of O substance we have obtained so far reveals that they still contain a small amount of a second component. Before a more detailed investigation into the chemical nature of the O substance can be profitably undertaken, therefore, it is considered advisable to await the isolation of an homogeneous preparation. It is, however, of interest to record at this stage of the investigation the results of the preliminary chemical observations, which certainly point to a close similarity in general make-up of the A and O substances. Both preparations contain about the same amount of nitrogen, hexosamine and amino-acids, and both substances yield similar quantities of reducing sugars and α-amino groups after acid hydrolysis. The specific rotation of the A and O substances are dissimilar, however, the former being weakly dextro-rotatory $[\alpha]_{5461} + 10° \pm 3°$, and the latter laevorotatory, $[\alpha]_{5461} - 23° \pm 3°$. The rotation given for the O substance must be considered provisional until an electrophoretically homogeneous preparation has been examined.

A specific and characteristic property shown by each of the blood group substances A, B and O is its capacity to react with *p*-dimethylaminobenzaldehyde reagent to yield an immediate reddish-purple coloration after treatment with dilute Na_2CO_3 at 100° for a few minutes. With the A and O substance the colour developed is equivalent to about 18 per cent of N-acetylglucosamine, and during the short alkaline hydrolysis the substances develop reducing properties equivalent to 4–5 per cent of glucose. The development of an immediate colour with Ehrlich's reagent after the specific substances have been treated with dilute alkali suggests that an oxazole ring structure is formed in a similar manner to the change which is known to occur when N-acetyl-glucosamine and other N-derivatives of glucosamine are similarly treated with dilute alkali (Morgan and Elson, 1934 ; Morgan, 1936, 1938). The alkali labile linkages in the O substance are most probably those glycoside linkages which join the first C atom of the N-acetylhexosamine molecules to other components of the O complex. The extreme alkali lability of the blood group substances seems to be a characteristic property, and has not been encountered during the examination of several complex polysaccharide substances which are known to contain hexosamine molecules (Morgan and King, 1943). It seems probable that some at least of the amino acid components are joined by glycoside linkage to the first C atom of the hexosamine, and that it is this linkage that is so extremely susceptible to the action of dilute alkali.

By means of simple inhibition tests employing a naturally occurring anti-O cattle serum or an immune anti-O rabbit serum as agglutinating agents, it has been possible to follow the purification of the O substance during its isolation from the cyst fluid and its destruction during hydrolysis with acid and alkali. The quantitative determination of the amount of group O substance in a solution by means of the inhibition test appears to be as reliable as similar determinations carried out for A and B substances. Results obtained by means of this test have shown that the most active A substance isolated from hog gastric mucin inhibits the agglutinating action of anti-O agglutinin on O erythrocytes as efficiently as the most potent O substance we have obtained from ovarian cyst fluids. It appears, therefore, that A substance derived from hog gastric mucin possesses both A and O serological specificity. In contrast with these findings the purest A-substance isolated from ovarian cyst fluids fails to inhibit the agglutination of O cells by anti-O serum, and therefore may be said to possess only A specificity. The A substance from human saliva behaves like the

hog mucin A substance in its power to inhibit the agglutination of O cells. This interesting serological relationship is being investigated further.

The specific blood group substances of human or animal origin (Morgan, 1944*a*) differ from the polysaccharide haptens of the bacterial somatic antigens (Morgan, 1944*b*) in that they possess firmly bound amino-acids which apparently form an integral part of each of the specific substances. The removal of the amino acids from the A complex with retention of full A specificity has not yet been accomplished, and it seems probable that the configuration of the amino-acid-containing component contributes to, or is entirely responsible for, the serological specificity of the complex. The presence of an amino-acid-containing component as part of the specific blood group complexes leads one to expect that these substances would possess antigenic properties *per se*, and indeed it has been found that purified A substance will induce, in a rather irregular manner, a limited but definite immune response in rabbits. The species of animal chosen for antigenic tests of this kind, however, is known to influence quantitatively the extent of the immune response, and as a result of some experiments carried out in collaboration with Dr. Loutit, it has been found that in man the injection of A substance of both animal and human origin gives rise to a more regular production of anti-A agglutinin than has been observed in the rabbit. It seems improbable that the presence of a small amount of a complete A antigen in the purified A substance is responsible for the observed activity, for it has been found that crude ovarian cyst fluids which show A activity are about as good antigenically, when measured under the same conditions, as is the purified A substance.

The present work shows that the specific O substance is antigenic in rabbits, and induces the formation of quite potent agglutinins reactive against O cells, whereas anti-A and anti-B agglutinins are formed to a much smaller extent, and human species agglutinins not at all. There seems little doubt that the O substance gives rise to a *specific* response, for it is known (Morgan and Watkins, 1944) that the administration to rabbits of the closely related A substance fails to induce the formation of anti-O agglutinins.

The stimulation of the natural anti-O agglutinins in cattle by means of the O substance is being studied, as is also the formation of a complete O antigen along the lines already developed for the A substance (Morgan, 1943; Morgan and Watkins, 1944).

SUMMARY.

(1) A polysaccharide-amino acid complex possessing blood group O specificity has been isolated from pseudomucinous ovarian cyst fluids.

(2) The O substance inhibits the agglutination of O erythrocytes by naturally occurring anti-O cattle serum and by immune anti-O rabbit serum.

(3) The agglutination of O cells by anti-O cattle agglutinin is inhibited by A-substance derived from hog gastric mucin, but not by A substance isolated from ovarian cyst fluids of human origin.

(4) Rabbits immunized with the purified O substance produce an anti-O immune serum, which, after absorption with A_1B erythrocytes, yields a potent anti-O reagent that is suitable for use in quantitative agglutination inhibition tests.

(5) The serological relationship and antigenic properties of the A and O substances are discussed.

Our thanks are due to Dr. R. A. Kekwick for examining the purified material in the Tiselius electrophoresis apparatus, and to Miss W. M. Watkins for much help with the immunological part of the investigation.

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FRACTURE METABOLISM AT DIFFERENT LEVELS OF PROTEIN INTAKE.

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Received for publication October 10, 1945.

IN recent years a considerable amount of attention has been devoted to the metabolic disturbances which follow injury. It has been established (Cuthbertson, 1930, 1931, 1932, 1936) that after an injury the patient has as a rule an increased output of urea and other nitrogenous substances in the urine. This rise in nitrogen (N) output is especially large following a fracture, and if the patient is kept on an ordinary hospital diet his N excretion for several weeks after the injury is often greater than his N intake (Howard, Parson, Stein, Eisenberg and Reidt, 1944). This leads to an extensive loss of N from the body (Cuthbertson, 1932), a loss which cannot be completely suppressed by feeding a diet rich in protein and calories (Cuthbertson, 1936).

The metabolic activities which lead to this N loss after injury and the bodily source from which the N is derived remain obscure, but several possibilities may be considered :

(*a*) A local origin, from the destruction of tissue by the injury and from disuse atrophy of the adjacent muscles. This question was studied in rats by Cuthbertson, McGirr and Robertson (1939). They killed their animals nine days after fracturing one femur, and compared the weights of the injured and uninjured limbs ; the loss of tissue indicated by this method was not sufficient to account for all of the excess N output in the urine. These authors point out, however, that an increase in the vascular bed and other local effects of the fracture may have masked a greater weight loss in the injured limb. Their observations cannot be regarded as conclusive proof that all the N lost after injury does not come from the injured limb.

(*b*) The N loss may occur because other tissues are raided in order to provide amino acids essential for repair. If the protein molecules of these tissues are disintegrated in order to produce the necessary amino acids, it is reasonable to assume that the non-essential amino acids will be discarded and appear as extra N in the urine. Croft and Peters (1945) think that this may explain the increased N output which occurs after burning. They find in rats that this extra N output can be much reduced by supplementing the diet with 1 per cent methionine, and they point out that methionine is needed in the production of new epithelium ; unless this is adequately provided in the diet, it may be necessary to raid the tissues for it.

(*c*) Labile protein reserves in the body (" deposit protein ") have frequently been postulated, and recently Kosterlitz and Campbell (1945) have described the conditions under which a labile cytoplasmic component of this type is formed in rat liver. They find that the amount of labile cytoplasm in the liver depends on the protein and calorie content of the diet ; it increases in quantity when the protein intake is high, and is readily lost when the protein or calorie intake is low. The extent to which such labile components of cytoplasm may contribute to the N lost after injury is not known.

We thought that some light might be shed on these alternative sources of N by a study of fracture metabolism at different levels of protein intake. The effect of a non-protein diet on the course of fracture metabolism has previously been reported in brief (Munro and Cuthbertson, 1943). Further results with this diet and with diets containing various amounts of protein are given in our present communication.

EXPERIMENTAL.

Animals.

Adult male and female rats were used, the total number studied being 118. Owing to war-time conditions four strains had to be employed, but care was taken to arrange the animals so that the different strains were represented on all levels of protein intake.

Diets.

The basic " non-protein " diet was :

Potato starch	23 parts
Rice starch	52 ,,
Cane sugar	10 ,,
Margarine	10 ,,
Salt mixture (De Loureiro, 1931) . . .	5 ,,

This diet contained 0·045 g. of N (equivalent to 0·3 g. of protein) per 100 g., and when casein (" ashless " extracted Glaxo) was added to make up the different levels of protein intake, equicaloric amounts of carbohydrate were removed. The protein

content of all diets was checked by N estimation. Three levels of casein intake were used in this study : (*a*) 0 per cent (i.e. the " non-protein " diet), (*b*) 10 per cent, and (*c*) 25 per cent casein. This last series is actually formed of groups of rats on slightly different levels (23–27 per cent), which average 25 per cent.

During each experiment the rats were given 2 ml. brewer's yeast extract (Chick and Roscoe, 1930) and 2 drops of cod-liver oil daily. The yeast extract was the main source of N in the non-protein diet experiments.

Feeding and urinary collection.

Since the rats were often untidy eaters, food was given to them only for two 1-hour periods in the day (9.30–10.30 a.m. and 3–4 p.m.), during which the cages were placed over paper. In this way scatter could be controlled, and food losses probably never exceeded 5 per cent of any single feed. Because of this feeding arrangement, urine could only be collected for 22 hours of the day, but the results recorded in this paper have been adjusted by calculation to a 24-hour basis starting from 9.30 a.m. each day. In most of the experiments we started urinary collection three days before fracture (as a control period), and ended it six days after fracture. The collection of excreta was carried out in the way described by Cuthbertson *et al.* (1939), faeces being separated by the Hopkins method. The faeces were not kept for examination because Cuthbertson (1930, 1936) had shown that there was no change in faecal N output as the result of fracture.

We have attempted to assess the accuracy obtained by this method of feeding and urinary collection. Eight rats were kept on the non-protein diet until the urinary output of N had reached a steady level. Various amounts of urea were then incorporated in the diet over 2-day periods. The N output during the administration of urea was compared with the N output of a control group to which no urea had been given ; the difference in N output represents the recovery of urea-N. In eight such experiments the average recovery was 97 per cent (standard deviation $\pm$ 10·9 per cent).

Urinary estimation.

Total N by macroKjeldahl method.

Fracture technique.

Fracture was inflicted under ether anaesthesia (duration 2–5 min.) by open operation on the left femur. The skin over the femur was incised and, by means of crushing forceps inserted into the wound, a comminuted fracture of the middle third of the shaft of the femur was produced along with a moderate amount of trauma to the adjacent muscle groups. The skin wound was then closed with a single suture. No wound became septic.

General procedure.

At the start of each experiment the rats were weighed fasting, and were then housed in metabolic cages in a thermostatically controlled room (maximum temperature range 23–27° C.). The specified diet was then fed to the group to be fractured or to a control group which was kept uninjured throughout the same period. At first the diet was given *ad libitum* and the animal allowed to satisfy its appetite. After the appetite had been gauged, this amount of food was given daily for the remainder of the experiment. In the case of a few animals there was a slight diminution of appetite after fracture ; this resulted in a small decline in N intake, which has been taken into account when assessing the effects of fracture on the N metabolism of these rats (see Table I).

TABLE I.—*N Balance after Fracture at Different Levels of Protein Intake.*

The data have been calculated per 100 g. of the initial body-weight of each rat.

Diet.	Number of animals.	Average body weight.		Pre-fracture period. (Average last 3 days.)			Post-fracture period. (Average of first 6 days.)			
		Initial.	Change in pre-period.	Total food intake.	N intake.	Urinary N.	Total food intake.	N intake.	Urinary N.	N balance.*
Fracture groups :		g.	%	g.	mg.	mg.	g.	mg.	mg.	mg.
0% casein	16	201	−10·8	5·2	8	14	5·2	8	15	−1·2
10% „	22	243	−1·1	5·1	80	56	4·9	79	64	−9·2
25% „	18	236	+6·2	5·2	215	157	5·2	214	182	−24·8
Control groups :										
0% casein	8	279	..	4·0	8	14·5	3·9	8	11·5	+2·8
10% „	6	265	+0·9	5·0	83	47	5·0	82	42	+4·2
25% „	4	286	+7·9	5·1	207	142	5·1	207	143	−1·1

* Mean daily N output of first six post-fracture days compared with mean daily N output of the three days preceding fracture. Comparison of similar periods for control groups.

RESULTS.

For the sake of uniformity, the data throughout the paper are expressed per 100 g. of the initial body-weight of the rat. Originally the data from male and female animals were analysed separately, but as this led to the same conclusions in both instances, we decided not to retain the sex division.

Fracture on 0 per cent, 10 per cent and 25 per cent casein diets.

The animals were fractured after they had been 12 days on the diet, and it was found (Fig. 1) that those on the non-protein diet showed only a slight and transient increase in N output; fracture on the 10 per cent level of casein intake caused a definite loss of N, and the loss was greater still on the 25 per cent casein diet.

More detailed treatment of these results is given in Tables I, II and III. In Table I the mean daily N output of the three days preceding fracture has been compared with the mean daily N output of the six days following fracture, the N intake being virtually constant over this period. The comparison confirms the data in Fig. 1, for it shows that the excess N output after fracture is least on the non-protein diet, rather more on the 10 per cent diet and greatest on the 25 per cent diet. The average N losses at these three levels of protein intake are significantly different from one another

TABLE II.—*Statistical Analysis of Data from Table I.*

N balance after fracture at different levels of protein intake.

Diet.	Number of animals.	Nitrogen balance after fracture. (Mean of first six post-fracture days.) N balance. mg.	Difference. mg.	"t" test.	P.
Fracture groups:					
0% casein	16	−1·2	8·0	3·26	<·01*
10% „	22	−9·2	15·6	4·03	<·01*
25% „	18	−24·8			
Control groups:					
0% casein	8	+2·8	1·3	0·52	·7–·6†
10% „	6	+4·1	5·2	1·53	·2–·1†
25% „	4	−1·1			

* Statistically significant.
† Not statistically significant.

(Table II). This contrasts with the behaviour of the control groups (Table I); their N outputs show only small fluctuations during the same period, and the fluctuations are not related to the level of protein intake (Table II). At all three levels of protein intake the N output of the fracture group after injury is significantly greater than the N output of the controls (Table III).

TABLE III.—*Statistical Analysis of Data from Table I.*

Comparison of fracture and control groups at different levels of protein intake.

Diet.	Number of animals. Fracture group.	Control group.	Nitrogen balance after fracture. (Mean of first six post-fracture days.) Fracture group. mg.	Control group. mg.	Difference. mg.	"t" test.	P.
0% casein	16	8	−1·2	+2·8	−4·05	2·86	<·01*
10% „	22	6	−9·2	+4·1	−13·32	4·01	<·01*
25% „	18	4	−24·8	−1·1	−23·71	2·78	·02–·01*

* Statistically significant.

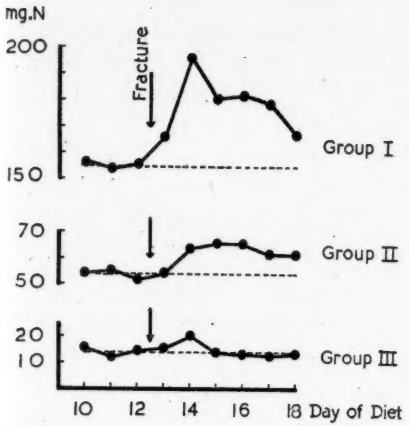

FIG. 1.

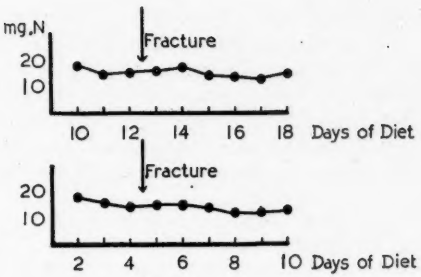

FIG. 2.

FIG. 1.—The effect of fracture on urinary N output at different levels of protein intake. Group I: 25 per cent casein diet (mean of 18 rats). Group II: 10 per cent casein diet (mean of 22 rats). Group III: 0 per cent casein diet (mean of 16 rats).

FIG. 2.—Urinary output of N as affected by fracture on different days of the non-protein diet: Upper group: Fracture on 13th day (mean of 8 rats). Lower group: Fracture on 5th day (mean of 8 rats).

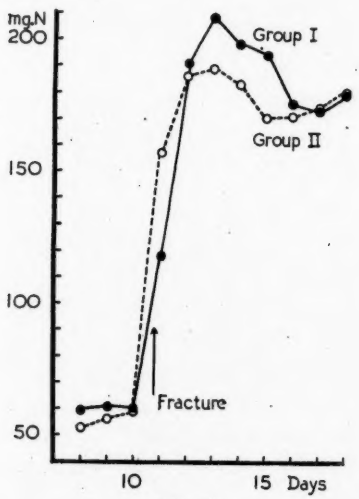

FIG. 3.

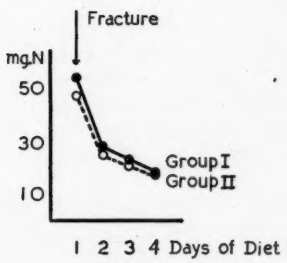

FIG. 4.

FIG. 3.—The effect on urinary N output of fracture inflicted on the day when the rats were changed from a 10 per cent casein diet to a 25 per cent casein diet. Group I: Fractured group (mean of 4 rats). Group II: Control group (mean of 4 rats).

FIG. 4.—The effect on urinary N output of fracture at the time of changing from a 25 per cent casein diet to a non-protein diet. Group I: Fractured group (mean of 10 rats). Group II: Control group (mean of 10 rats).

Day of fracture on the non-protein diet.

It may be wondered whether fracture earlier than the 13th day of the non-protein diet might have caused a more definite rise in N output than that shown in Fig. 1. However, Fig. 2 demonstrates that fracture on the fifth day of the diet produces an equally small response.

Influence of the protein intake after fracture.

It is obvious from our results that the metabolic response to fracture is influenced by the protein content of the diet. In the experiments so far described, the protein content of the diet has been the same before and after fracture. This arrangement does not allow us, however, to decide whether the effect depends on the protein intake before fracture or on the intake after fracture. This question was studied by changing the protein intake on the day of fracture (*a*) from a low to a higher level, (*b*) from a high to a low level.

(*a*) Two comparable groups of four animals each were put on the 10 per cent casein diet, of which they ate daily 5·6 g. per 100 g. body-weight. On the 11th day of the experiment one group had their left femora broken, while the other group were only anaesthetized. Both groups were then transferred to the 25 per cent casein diet, the total daily quantity of food eaten being the same as before fracture. A comparison of the N excretions of the fractured and control groups (Fig. 3) shows that the N loss after fracture is much less than we had noted in animals receiving the 25 per cent casein diet throughout the experiment (Fig. 1). The extra N excretion of the fractured group during the first six post-fracture days is 8·9 mg. per 100 g. weight per day; this figure is slightly less than the extra N output of the fractured group kept on the 10 per cent casein diet throughout the experiment (Table III).

Thus it would appear that the greater N loss at the 25 per cent level of protein intake (Fig. 1) is entirely dependent on the rats having had this level of casein intake during the period preceding fracture.

(*b*) Two similar groups of 10 rats each were fed the 25 per cent casein diet, on which they gained weight slightly. They were then changed to the non-protein diet; one group was fractured on the day on which the change was made, the other group being kept uninjured as controls. In each group the N output in the urine declined at approximately the same rate (Fig. 4); during the first four days of the non-protein diet the two groups excreted respectively 125·9 and 113·0 mg. N in the urine per 100 g. of their initial body-weight. The extra output of the fractured group (3·2 mg. N. per day) is not significant (Fisher's " t " test = 1·02); when the injury is inflicted on the 13th day of the non-protein diet, a somewhat similar difference between the N outputs of fractured and control groups is found (Table III).

This indicates that the influence of the 25 per cent casein diet on fracture metabolism is lost as soon as the protein intake is reduced.

Influence of body-weight changes.

Although the animals taking the 25 per cent casein diet throughout the experiment (Fig. 1) had somewhat variable N losses after fracture, we noticed that the rats which had failed to gain weight before injury generally had a smaller N loss after injury (Table IV). Indeed the greater N output at the 25 per cent level of intake as compared with the 10 per cent level of intake (Fig. 1) seems to depend to a large extent on the animals having had an increase of weight on the 25 per cent diet. At the 10 per cent level of casein intake there does not appear to be any relationship between change in weight before fracture and N output after fracture (Table IV).

TABLE IV.—*Statistical Analysis of Data from Table I.*

Change in body-weight during the pre-fracture period in relation to nitrogen balance after fracture.

Change in weight during pre-fracture period.	10% casein diet.			25% casein diet.			
	Number of rats.	N intake. mg.	N balance. mg.	Number of rats.	N intake. mg.	N balance. mg.	
−10% upwards	0	..	..	6	239	−29·8	−32·5*
+5% to +9·9%	3	77	−8·6	4	214	−36·4	
nil to +4·9%	5	80	−9·5	4	187	−18·5	−16·1*
−0·1% and less	14	81	−9·3	4	209	−13·4	

* Difference = 16·4 mg.; Fisher's "t" test = 2·13; P = 0·02–0·05, i.e. the difference is significant.

Presumably those animals on the 25 per cent diet which did not gain weight had failed to adjust their appetites to their caloric requirements; certainly there is no evidence (Table IV) that their N intakes were much lower than the N intakes of the other rats on this diet.

DISCUSSION.

When rats on a non-protein diet receive a fracture, they respond with a slight rise in N output; rather more N is lost when animals on a 10 per cent protein diet have a limb fractured, and on a 25 per cent diet the loss is greater still (Fig. 1, and Tables I, II, and III). Thus the amount of N lost after injury depends on the level of protein intake.

If the animals are transferred from the 10 per cent to the 25 per cent protein diet *after* fracture, there is no greater N loss than one finds when the animals are on the 10 per cent diet throughout (Fig. 3). Therefore the large N loss associated with the 25 per cent protein intake depends on the feeding of this diet during the pre-fracture period. Furthermore, the influence of the 25 per cent protein diet is greatest when the animal gains weight on this diet in the pre-fracture period (Table IV), and it is immediately lost when the rat is put on a non-protein diet (Fig. 4). Thus some change is produced during the pre-fracture period which is readily reversed under adverse nutritional conditions.

Unless one imagines that the tissues of the injured limb are remarkably sensitive to protein intake, one cannot explain our results simply as due to local tissue destruction and atrophy at the site of injury. It is equally unlikely that the N loss is due to raiding of tissues for essential amino acids; the largest losses occurred on the diet containing 25 per cent of protein (Fig. 1). Instead, one must look for some metabolic process which adapts itself readily to changes in protein intake. It is therefore interesting to note that the conditions under which Kosterlitz and Campbell (1945) found labile liver cytoplasm to accumulate to the greatest extent are those under which we have found that the animal will lose most N if it receives an injury. This labile cytoplasm is quickly lost on a non-protein diet, and coincident with its disappearance goes the ability to lose N after injury (Fig. 4). Clearly all the N lost after injury cannot come from the labile stores of the liver, especially in human cases, where the loss is often prolonged and extensive. It may be that other labile resources in the body are extensive, or that protein liberated by local tissue damage is used more economically when the protein stores are low. Further investigations of the metabolic changes occurring after injury are in progress.

SUMMARY.

1. The changes in N output which follow fracture of a rat's femur have been investigated at different levels of protein intake, viz. 0 per cent, 10 per cent and 25 per cent.

2. After fracture the rats on the non-protein diet have a slight and transient loss of N ; a larger loss is observed with animals on the 10 per cent protein diet, and on the 25 per cent diet the loss is greater still. This greater N loss at the 25 per cent level of intake depends on the rat having gained weight during the pre-fracture period.

3. If the femora of rats on the 10 per cent. protein diet are broken and the animals are then transferred to the 25 per cent protein diet, the N loss is similar to that obtained when the 10 per cent diet is used throughout, and is much less than the N loss observed when the 25 per cent diet has been eaten throughout.

4. It is concluded that the amount of N lost after injury is modified by the level of protein intake during the pre-fracture period. The effect of feeding a high protein diet during the pre-fracture period is lost if the animal is transferred to a non-protein diet after fracture, and it is suggested that our findings are due to changes in the amount of labile (deposit) protein in the body at different levels of protein intake.

We wish to take this opportunity of thanking Profs. Cathcart and Wishart and Dr. Cuthbertson for their interest in this work. One of us (H. N. M.) is indebted to the Medical Research Council for a grant for technical assistance.

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PRODUCTION OF ANTIBIOTICS BY FUNGI.

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Received for publication November 3, 1945.

In searching for antibiotics of microbial origin, the culture fluids of a large number of fungal genera and species have been examined for the production of antibacterial substances. A few of the results differ apparently from those of Wilkins and Harris (1943), doubtless owing to the use of different strains and culture conditions.

METHODS.

Fungi were grown in conical flasks (150 ml.) containing 40 ml. of nutrient solution. In some experiments, such as in the isolation of kojic acid, the moulds were also cultured in batches of quart-size milk bottles each containing 400 ml. of nutrient solution. Cultures were usually incubated at 24° C., though some fungi grew more satisfactorily at 20° C.

The following media were used :

(1) 7·5–8 per cent Corn-steep liquor together with glucose (usually 4 per cent). This medium was as satisfactory as similar ones containing additional salts which were used in initial experiments.

(2) 1·0 per cent Bacto-tryptone with varying amounts of glucose.

(3) Czapek-Dox type media. Sodium nitrate, 2·0 g.; potassium chloride, 0·5 g.; magnesium sulphate heptahydrate, 0·5 g.; ferrous sulphate heptahydrate, 0·01 g.; dipotassium phosphate, 1·0 g.; glucose, 40 g. unless otherwise indicated; water to 1000 ml. In most cases Difco yeast extract, 1 g., was added.

Routine tests for antibacterial activity were made either by the agar plate method or by serial broth dilutions; results obtained by the former method were at intervals related to the serial dilution figures by concurrent assays. In the dilution assays a peptone-lemco (Lab-Lemco, Oxo Ltd.) broth was used. Inoculations were made with 18–24-hour cultures of *Staphylococcus aureus* as the primary organism employed in tests for antibacterial activity. The degree of inhibition was read after 18 hours' incubation at 37° C. Fluids giving positive results were tested against a range of other bacteria drawn from both the Gram-positive and Gram-negative groups, including several plant parasitic species (*cf.* Waksman, Bugie and Reilly, 1944). *Mycobacterium phlei* was included in many of the tests as representative of the acid-fast group of bacteria. As *Myco. phlei* grows only slowly, cultures which were 3–4 days old were used as inoculum in broth dilution tests and results read after 2, 3, and up to 7 days' incubation at 37° C.

RESULTS.

General.

Excluding some *Aspergillus* spp., cultures of the following fungi* were inactive against *Staph. aureus*: *Verticillium albo-atrium*, *V. dahliae*, *Monascus ruber*, *Eremascus fertilis*, *Nematospora gissipii*, *Melanospora zamiae*, *Sordaria* sp., *Podospora* sp., 3 *Alternaria* spp., 3 *Rhizopus* spp., a number of *Mucoraceae* spp., 1 culture of a *Chaetomium* sp., 4 *Fusaria* strains, and some unidentified moulds obtained from rotting plant material.

Antibacterial activity was found in the culture fluids of 2 strains of *Chaetomium* sp. and the allied genera *Philocopra*, *Melanospora destruans*, *Trichoderma* sp., *Botrytis cinerea* and 5 species of *Fusarium*, in addition to several species of *Aspergillus*, in particular *A. parasiticus*, with which much of the following work was concerned.

Aspergillus sp.

Strains of the following species of *Aspergillus* were tested against *Staph. aureus* for antibiotic activity on various media. *A. parasiticus* (4 strains), *A. flavus* (4 strains), *A. tamarii* (2 strains), *A. niger*, *A. oryzae*, *A. ruber*, *A. repens* and *A. chevaliere* (1 strain each).

The last four species and one of the strains of *A. tamarii* gave no activity, while the second strain of *A. tamarii* gave rise to only slight activity. Of the strains of *A. flavus* one was inactive, a second produced very feeble activity, and the remaining two exhibited considerable antibacterial activity. The maximum titre (1 : 200) was reached about the 4th day, but a rather rapid fall in potency of the culture fluid occurred after the 5th day. All four strains of *A. parasiticus* (AC 14, NCTC 975, A 13 and A 4) produced culture filtrates which were active, but of these AC 14 was decidedly the most effective and was used in all the later experiments.

* Our thanks are due to Prof. H. Raistrick, F.R.S., and to Dr. L. E. Hawker, from whom many of the fungal cultures were obtained.

Production of antibiotics by A. parasiticus (*AC* 14).

The production of an antibiotic resembling penicillin (" parasiticin ") by *A. parasiticus* has been briefly described (Cook and Lacey, 1944). This material has been prepared in fair quantity, and its properties and relation to authentic penicillin will be recorded in another communication.

Parasiticin was produced on media containing corn-steep liquor as an essential constituent, but when *A. parasiticus* was grown on Czapek-Dox medium containing 4 per cent glucose and 0·1 per cent yeast extract, it developed antibacterial activity most of which was due to formation, not of parasiticin, but of kojic acid (Cook and Lacey, 1945 ; *cf.* Jennings and Williams, 1945). The isolated material (Birkinshaw *et al.*, 1931) completely inhibited the growth of *Staph. aureus*, *Ps. pyocyanea*, and *Bact. coli* at a limiting dilution of 1 : 2000, but accounted for only 25–30 per cent of the original activity. However, the selective antibacterial activity of culture solutions on synthetic media varied with the amount of sugar used. Thus in media containing 0·5 or 1 per cent glucose, activity against *Staph. aureus* appeared on the 21st day and the solutions remained active for 3–4 weeks ; throughout this period the ferric chloride test for kojic acid was negative, and the solutions were entirely ineffective against the Gram-negative organisms *Bact. coli* and *Ps. pyocyanea*. Cultures in synthetic media with 2 per cent glucose contained kojic acid after 5–7 days, and were slightly active against the above Gram-negative organisms as well as against *Staph. aureus*. Activity then disappeared, but was regained on about the 13th day, when the solutions were active against *Staph. aureus* but not against *Bact. coli* or *Ps. pyocyanea* ; during this phase tests for kojic acid were negative. A similar phenomenon was observed when the medium contained 4 per cent glucose. Activity against *Staph. aureus* appeared on about the 5th day, reached a maximum on the 11th day and then fell sharply to zero, but the culture fluids again became active on the 25th day and were still potent on the 50th day. During the first phase of activity relatively high concentrations of kojic acid were present, and the solutions were active not only against the Gram-positive organisms *Staph. aureus*, *B. megatherium* and the plant parasite *Bact. fascians*, but also against the Gram-negative organisms *Bact. coli*, *Ps. pyocyanea*, *Chr. prodigiosum*, *Ps. fluorescens liquefaciens*, *Bact. aerogenes* and *B. carotovorus*. During the second phase, when kojic acid was no longer present, the growth of Gram-positive organisms only was inhibited, the maximum titre against *Staph. aureus* being about 1 : 100.

When 1, 2 or 4 per cent lactose or maltose was used in place of glucose in the synthetic media, all three of the lactose cultures and also those in 1 or 2 per cent maltose were similar to cultures in 1 per cent glucose in antibiotic production ; in 4 per cent maltose solutions antibacterial activity exactly paralleled that in 4 per cent glucose solutions, kojic acid being first formed and later destroyed. This dual phase of activity invariably occurred in a number of experiments in which substitutes for yeast extract (without which production of antibiotics was less pronounced and considerably delayed) were added to Czapek-Dox medium containing 4 per cent glucose ; the two phases, however, were not always so distinct as in cultures in yeast extract. Freshly prepared extract of bakers' yeast and an extract of lettuce seedlings were effective supplements, but individual compounds such as aneurin, lactoflavin, pantothenic acid, pyridoxine, *p*-amino-benzoic acid, or heteroauxin were almost valueless in increasing antibiotic production.

It may be concluded therefore that in these synthetic media *A. parasiticus* is capable of producing at least two antibiotics, one of which is kojic acid.

Antibacterial activity of A. parasiticus *against* Myco. phlei.

During the above experiments, culture fluids of *A. parasiticus* grown on corn-steep liquor with glucose or on synthetic media were examined for any ability to inhibit the growth of *Myco. phlei* (as representative of the acid-fast bacteria), invariably with negative results. When however the mould was grown in 1 per cent. Bacto-tryptone solution, or on a medium containing corn-steep liquor without the addition of sugar, an antibiotic was produced which was markedly active against both *Staph. aureus* and *Myco. phlei*, and to a lesser degree against *B. subtilis*, *Strept. pyogenes*, *Bact. coli* and the plant parasites *Bact. tumefaciens* (Gram-negative) and *Bact. fascians* (Gram-positive), but not against *Ps. pyocyanea* or *Ps. fluorescens liquefaciens*. In further experiments flasks containing either 1 per cent Bacto-tryptone, or 0·5 per cent Bacto-tryptone plus inorganic salts, with varying amounts of glucose were inoculated with *A. parasiticus* and incubated at 25° C. In all cases appreciable activity against *Myco. phlei* appeared only in the absence of glucose. Activity against *Staph. aureus* in the cultures containing glucose was similar to that obtained from *A. parasiticus* on purely synthetic media with varying amounts of glucose. Kojic acid was abundant in the 4 per cent glucose cultures after 7–11 days, but both kojic acid and activity had disappeared by the 17th day; the fluids throughout were inactive against *Myco. phlei*. Bacto-tryptone culture fluids containing 1 or 2 per cent glucose developed considerable activity against *Staph. aureus* which was not attributable to kojic acid, but were inactive against *Myco. phlei* except for a very slight and transient activity at 16–18 days.

In 1 per cent Bacto-tryptone without glucose titres of 1/50 against *Staph. aureus* and of 1/80 against *Myco. phlei* were reached in 4–6 days, after which activity against *Staph. aureus* decreased, whereas that against *Myco. phlei* remained substantially constant until the 14th day, when a further small increase in strength was maintained as long as observations were made (38 days). Results in 0·5 per cent Bacto-tryptone with additional inorganic salts were similar, except that potency against *Myco. phlei* was lower and delayed when compared with that in 1 per cent Bacto-tryptone.

These experiments indicate that *A. parasiticus* may produce at least 4 antibiotics under varying cultural conditions: (i) parasiticin which resembles penicillin, (ii) kojic acid, (iii) an antibiotic produced in old cultures on synthetic media containing 4 per cent glucose following the disappearance of kojic acid, and (iv) an antibiotic effective against *Staph. aureus* and *Myco. phlei* produced in corn-steep liquor or Bacto-tryptone media without added carbohydrate.

Soltys (1944) has recently reported a substance (" aspergillin ") from *A. fumigatus* which inhibits the growth of acid-fast bacteria including *Myco. phlei* but not of *Staph. aureus*. Again, Kallos (1945), working with an unidentified strain of *Aspergillus*, obtained an antibiotic which was active against *Myco. tuberculosis* and *Staph. aureus*. This material was believed to be not identical with gliotoxin, helvolic acid or patulin, and was produced on synthetic media containing glycerine and nitrogenous substances such as asparagine, but not on Czapek-Dox medium. It is not unlikely that the inhibitory substance produced by *A parasiticus* on Bacto-tryptone media and which is active against *Staph. aureus* and *Myco. phlei* may be related to the material obtained by Kallos.

Chaetomium *and related genera.*

Waksman and Bugie (1944) reported the discovery of an antibiotic (chaetomin) produced by *Chaetomium cochliodes*. This material was not obtained from other *Chaetomium* species or from other strains of *Chaetomium cochliodes* which they examined.

In investigations made before the publication of Waksman and Bugie's results, we had also obtained an antibiotic resembling chaetomin in its antibacterial activity from a *Chaetomium* of unknown species. The best results were obtained in Czapek-Dox medium containing 4 per cent glucose and 0·1 per cent yeast extract, pH = 5·5–6 ; 22°–25° C. incubation. The crude culture fluids caused complete inhibition of *Staph. aureus* at broth dilution of 1 : 100, and were also markedly active against *B. megatherium* and the Gram-positive plant parasite *Bact. fascians*. Partial inhibition of *Bact. coli* and *Ps. fluorescens liquefaciens* was also observed, but other Gram-negative bacterial species (*Ps. pyocyanea*, *Chr. prodigiosum*, *Bact. aerogenes*, *Bact. tumefaciens*, *B. carotovorus* and *B. phytophthorus*) were unaffected. A strain of *C. cochliodes* produced metabolism solutions which were similarly active though less potent, but a second strain of this species was inactive. Culture fluids of strains of *Philocopra* sp. and *Melanospora destruans* also inhibited the growth of *Staph. aureus*, but cultures of *Melanospora zamiae*, *Sordaria* sp. and *Podospora* sp. were inactive.

Trichoderma sp.

The antifungal action of *Trichoderma* sp. against *Rhizoctonia*, *Pythium*, *Botrytis cinerea*, etc., has been observed by several investigators. Weindling (1938, 1941) found that the active principle causing fungal inhibition was very unstable and transient ; thus the activity against *Rhizoctonia* in *Trichoderma* cultures was at a maximum after 48 hours and quickly disappeared thereafter. On the other hand, Brian (1944) has observed formation of the more stable gliotoxin by some strains of *Trichoderma viride*, and Brian and McGowan (1945) found that a substance (viridin) of high fungistatic activity, but not markedly bacteriostatic, was produced by other *T. viride* strains.

During the present work antibacterial activity was observed in the culture fluid of a *Trichoderma* sp. (isolated from soil) which resembled the antifungal activity obtained by Weindling in its transient character ; the maximum activity was found in cultures 4–7 days old, which then rapidly became devoid of inhibitory action. The antibacterial culture fluids were active against the Gram-positive *Staph. aureus*, *B. megatherium* and *Bact. fascians*, but not against *Bact. coli*, *Ps. pyocyanea* and other Gram-negative species.

Botrytis cinerea.

A lettuce strain of *B. cinerea* grown on a corn-steep liquor medium or on a Czapek-Dox medium with the addition of yeast extract produced slight antibacterial activity, not only against *Staph. aureus*, but also against the Gram-negative organisms *Bact. coli*, *Ps. pyocyanea*, *Bact. tumefaciens* and *B. carotovorus* ; it was inactive against *Ps. fluorescens liquefaciens* and *Bact. aerogenes*. This activity, however, was very transient, like that of *Trichoderma*. The age of the culture fluids when it first appeared varied with the type of medium, but the maximum titre, reached on the 10th to 13th day in corn-steep media, was invariably maintained for not more than 48 hours, after which there was a rapid fall in activity.

Fusaria.

Five *Fusarium* strains among the fungi isolated from diseased plant material were examined for antibacterial activity. Of these, two were completely inactive but three very slightly inhibitory towards *Staph. aureus*. Further tests with the most promising (later identified by Dr. W. L. Gordon, Winnipeg, as *F. sambucinum*) showed that a Bacto-tryptone-glucose medium was preferable to a purely synthetic one, but

that antibacterial activity of the culture fluids was similar in both types of media. The fluids were more active against *Myco. phlei* than against *Staph. aureus*; for example, a culture fluid which completely inhibited the growth of *Myco. phlei* at a broth dilution of 1 : 25 was effective against *Staph. aureus* at a limiting dilution of only 1 : 10.

This result led to the examination of four more *Fusarium* species, *F. culmorum*, *F. dianthi*, *F. fructigenum* and *F. javanicum*, which were also cultured in synthetic and Bacto-tryptone media with varying amounts of glucose. The first two species gave uniformly negative results, *F. fructigenum* showed slight antibiotic activity similar in character to that of *F. sambucinum*, but *F. javanicum* when cultured in a Bacto-tryptone medium (sodium nitrate, 2·0 g.; potassium chloride, 0·5 g.; magnesium sulphate heptahydrate, 0·5 g.; ferrous sulphate, 0·01 g.; dipotassium phosphate, 1·0 g.; Bacto-tryptone, 5 g.; glucose, 40 g.; distilled water to 1,000 ml.) produced strongly antibacterial metabolism solutions. The maximum titre was produced after 8–12 days and was maintained with but little loss of potency for at least 3–4 weeks; the solutions were effective against both *Staph. aureus* and *Myco. phlei* at a limiting dilution of about 1 : 100. They were equally active against *B. subtilis* and the Gram-positive plant parasite *Bact. fascians*, less active against *Strept. pyogenes*, very slightly active against *Bact. coli*, and inactive against *Ps. pyocyanea* and *Ps. fluorescens liquefaciens*. No antibacterial activity was observed in Bacto-tryptone media lacking glucose or in a purely synthetic medium. The examination of the antibacterial substances produced by *F. sambucinum*, *F. javanicum* and other *Fusarium* species as well as others mentioned in earlier sections is being continued.

SUMMARY.

Inhibitory actions, often of varying kinds and degrees of permanence, towards a range of Gram-positive and negative bacteria including several plant parasites have been found in cultures of *Aspergillus*, *Fusarium*, *Chaetomium*, *Trichoderma* and other minor species.

Differential estimates of antibiotic effectiveness indicated that *A. parasiticus* was able to produce at least four antibiotics, including kojic acid and a penicillin-like material.

Several *Fusaria* produced solutions active against *Myco. phlei* as well as other organisms.

We thank Professor I. M. Heilbron, D.S.O., F.R.S., for his interest and encouragement during this work, and the Rockefeller Foundation for financial assistance.

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THE INFLUENCE OF TEMPERATURE ON THE ADAPTATION OF "TETRATHIONASE" IN WASHED SUSPENSIONS OF *BACT. PARATYPHOSUM B.*

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Received for publication November 12, 1945.

ALTHOUGH it has been known for some time that the enzyme activities of washed suspensions of micro-organisms vary according to their method of preparation, composition and pH of culture medium, duration of growth, etc. (see Gale, 1943), it is only recently that clear evidence has been obtained that an increase in enzyme activity can occur in the absence of cell multiplication. Examples of such enzyme "adaptation" without growth have been reported for galactozymase of *S. cerevisiae* (Dienert, 1900; Stephenson and Yudkin, 1936), formic hydrogenlyase of *E. coli* (Stephenson and Stickland, 1933), galactozymase of *Strep. lactis* (Hegarty, 1939), an enzyme oxidizing *p*-amino-benzoic acid in a soil bacillus (Mirick, 1943), and "tetrathionase" of *Bact. paratyphosum B* (Knox and Pollock, 1944). Even in cases where cell growth occurs there is evidence that the factors most favouring growth are not necessarily those which result in highest activity for individual enzymes in the resulting cell suspensions, and the largest yield of active enzyme is often obtained under conditions which give relatively poor growth. There is a similar dissociation between adaptation and the rate of the enzyme reaction itself; an environment which favours the "formation" of an enzyme is not necessarily that at which the enzyme reaction proceeds at a maximal rate, and vice versa. Previous work on this subject has been reviewed by Gale (1943).

Although, therefore, the process of enzyme adaptation is to a large extent independent of both growth and enzyme action, there has so far been no attempt to investigate its mechanism under conditions where both these complicating factors could be adequately controlled.

The enzyme system "tetrathionase," which is responsible for the reduction of sodium tetrathionate to thiosulphate, is particularly suitable for the study of enzyme adaptation for three reasons:

(*a*) The enzyme reaction can be followed simply and accurately by direct iodometric titration of thiosulphate formed.

(*b*) Enzyme adaptation occurs rapidly in the presence of buffer, mannitol and tetrathionate without addition of a source of N and in the absence of cell multiplication or increase in cell protoplasm.

(*c*) Washed suspensions, once adapted, retain their heightened activity relatively unaltered, no significant decrease or further increase occurring. It is thus possible to measure the effect of various factors upon the enzyme action alone by using suspensions which have already completed their adaptation, and differ from those employed for studying adaptation only by the fact that they have been previously treated for 2–3 hours with mannitol and tetrathionate.

It was hoped that it might be possible to study, more accurately than hitherto, some of the factors influencing the adaptation process. The present paper is concerned with the effect of temperature.

* A report to the Medical Research Council.

METHODS.

The organism used was *Bact. paratyphosum B* (3 different strains). Suspensions were prepared from 18 hours' growth on nutrient agar at 37°. Tetrathionate reduction was followed by recording the values of the iodine titration in samples of a suspension mixture containing M/50 mannitol, M/50 tetrathionate and M/5 phosphate buffer pH 7·8, as previously described (Pollock and Knox, 1943). "Adapted" suspensions are those which have been treated at 37° with M/50 mannitol, M/50 tetrathionate and M/5 phosphate buffer for 2–3 hours until the rate of reduction is approximately constant, and then washed twice with $\frac{1}{4}$-strength Ringer's solution (see Knox and Pollock, 1944).

The reaction was followed at different temperatures in thermostatically controlled water baths.

Viable counts were estimated by the pour-plate method, using duplicate dilutions in broth or $\frac{1}{4}$ strength Ringer's solution and 3 plates for each dilution series, i.e. 6 plates per count.

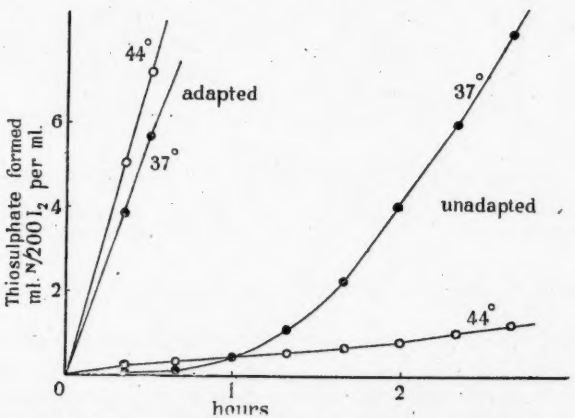

FIG. 1.—Comparison of effects of temperature on tetrathionate reduction by unadapted and by adapted suspensions of *Bact. paratyphosum B*.

RESULTS.

The rate of tetrathionate reduction using fully adapted suspensions is constant for at least several hours, and a comparison of the rates at 37° and 44° showed, as might be expected, that the reaction was faster, by 30–40 per cent, at 44°. With unadapted suspensions, however, an entirely different picture was seen (Fig. 1). The initial speed at 44° was slightly greater than at 37°, but little increase in the rate occurred at the higher temperature over a period of 2 or 3 hours, whereas at 37° there was a great increase in enzyme activity. Whatever factor or factors were responsible for increasing enzyme activity at 37°, they did not come into operation at 44°.

This inhibition of adaptation at 44° was confirmed by the next experiment, in which an unadapted suspension was divided into four parts and treated at 44° and 37° with and without tetrathionate for $2\frac{1}{2}$ hours, each part being then washed, re-suspended and tested at 37° for ability to reduce tetrathionate (see Fig. 2). Although treatment at 44° caused some permanent loss in ability to be adapted, the increase in the power to reduce tetrathionate resulting from treatment with the substrate at 44° (curves C and D) was insignificant in comparison with the increase resulting from

similar treatment at 37° (curves A and B). In other words, treatment with tetrathionate at 37° resulted in a fully adapted suspension (curve A); but treatment with tetrathionate at 44° resulted only in a very slight increase in the rate of tetrathionate reduction compared with a suspension treated at that temperature without tetrathionate.

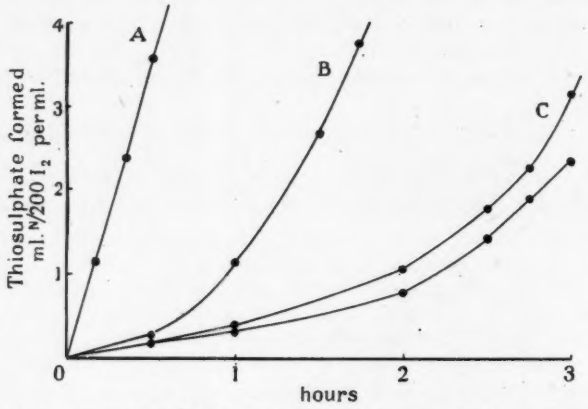

FIG. 2.—Tetrathionate reduction at 37° by suspensions of *Bact. paratyphosum B* previously treated for 2½ hours as follows:
A. With tetrathionate } at 37°.
B. Without tetrathionate }
C. With tetrathionate } at 44°.
D. Without tetrathionate }

There are, however, two possible objections which have to be answered:

(1) *The higher temperature* (44°) *may cause gradual inactivation of the enzyme system.*

There is, of course, no question of the higher temperature directly inhibiting the reduction of tetrathionate. This was clearly shown (Fig. 1) by the increased rate of tetrathionate reduction at 44° in adapted suspensions; moreover, this relative increase was maintained over several hours (not shown in Fig. 1). If, however, there were a slow progressive inactivation of enzyme, the apparent failure of the organisms to become adapted at 44° might be due to simultaneous inactivation and adaptation. That this was not so is shown in Table I, where the activities of 2 portions of a previously adapted suspension, treated for 3 hours at 37° and 44° respectively in M/5 phosphate buffer and subsequently investigated at 37°, show no significant difference.

TABLE I.—*Effect of Treatment of Adapted Suspension of* Bact. paratyphosum B *for 3 hours at 37° and 44°.*

Temperature of treatment.	Subsequent rate of tetrathionate reduction at 37° (ml. N/200 I_2 per ml. per 30 min.).
37°	5·3
44°	5·15

(2) *There might be an absolute or relative increase in the number of viable organisms at* 37°.

That there is no absolute increase in the viable count during the adaptation process at 37° has been clearly shown in a previous paper (Knox and Pollock, 1944). The

possibility, however, exists that, although at 44° adaptation is taking place, its effects are masked by a simultaneous decrease in viable cells. For instance, it is apparent from Fig. 2 that treatment at 44° permanently impaired the ability of the organisms to adapt themselves at 37°, and if that damage were associated with a large reduction in the viable count, the apparent inhibition of adaptation at 44° might be explained on the hypothesis that nonviable cells cannot become adapted.

Repeated experiments, however, show that the decrease in the viable count during treatment with tetrathionate at 44° is always small and never greater than at 37°. Table II shows the results of two experiments in which the numbers of living organisms were counted before and after treatment with M/50 mannitol, M/50 tetrathionate and M/5 phosphate buffer at 37° and 44°. There is no significant difference between the counts at the two temperatures, although at 37° the suspensions had, at the end of the experiment, become fully adapted.

TABLE II.—*Viable Counts of Suspensions Before and After Treatment with Tetrathionate at* 37° *and* 44°.

	Initial count (viable cells/ml. × 10^9).	Final count. 37°.	Final count. 44°.	Duration of experiment.
Experiment 1	38·5	31·2	31·0	3 hours
,, 2	27·9	20·7	21·1	2 ,,

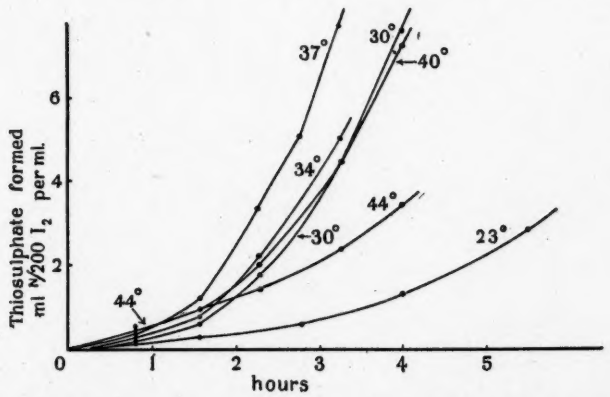

FIG. 3.—Effect of temperature on tetrathionate reduction by unadapted suspensions of *Bact. paratyphosum B.*

Since the rate of adaptation at 44° is negligible compared to that at 37°, it follows that there must be an optimum temperature for the process. Fig. 3 shows curves obtained with unadapted suspensions at temperatures from 23° to 44°. These curves are, of course, the result of the combined effect of temperature on both enzyme adaptation and the enzyme reaction itself. The picture in Fig. 3, where curves cross over each other, is therefore at first sight somewhat confusing. It is, however, easy to understand if it is assumed, as indeed appears to be the case, that there is a small amount of active enzyme even in the completely unadapted suspensions at the beginning of the experiment. Initially, therefore, the predominant effect of temperature would be on the enzyme reaction; later, when adaptation had proceeded for

some time, the effect on the enzyme system would be overshadowed by the effect on adaptation, which is far more striking. Although, during the first hour of adaptation, the titrations are small, this " crossing-over " effect is a consistent and significant phenomenon. For instance, at 40° the initial rate of tetrathionate reduction is higher than at 30° or 37°, but later falls below both the 37° and 34° rates and finally even below the rate at 30°.

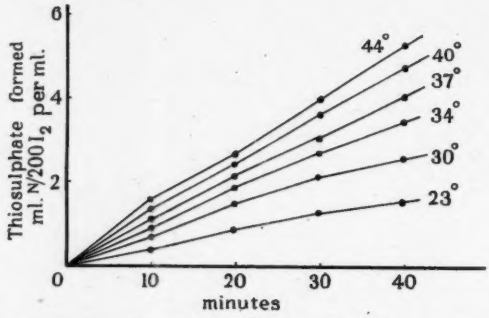

FIG. 4.—Effect of temperature on tetrathionate reduction by previously adapted suspensions of *Bact. paratyphosum B*.

The effect of temperature on the rate of action of " tetrathionase " alone is shown in Fig. 4, using fully adapted suspensions. These suspensions were samples from the same bulk suspension used in the experiment with unadapted cells illustrated in Fig. 3, and differ only in that they were previously treated at 37° with tetrathionate

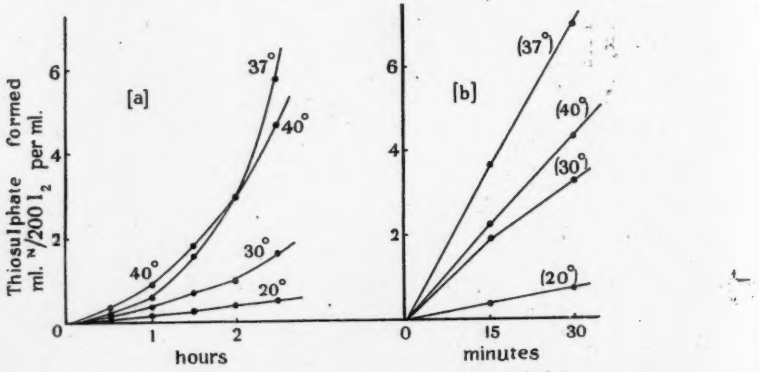

FIG. 5.—Proportions of active enzyme present in suspensions of *Bact. paratyphosum B* after adaptation for 2½ hours at different temperatures.
(*a*) Tetrathionate reduction during adaptation at different temperatures.
(*b*) Tetrathionate reduction at 44° after adaptation for 2½ hours as in (*a*). (Temperatures marked are those of previous adaptation.)

and mannitol for 2–3 hours, with subsequent washing. The expected progressive increase in the rate of reaction with rise in temperature is seen to occur.

The effect of temperature on adaptation can be illustrated more clearly if samples of the suspension are adapted at different temperatures for the same period of time,

and are then washed and resuspended and their activities measured at 44°. Little further adaptation can occur at this temperature, and it is thus possible to estimate directly the relative amounts of active enzyme present. Fig. 5 (*a*) shows the adaptation curves of suspension samples incubated with tetrathionate and mannitol in the usual way at 20°, 30°, 37° and 40°; after 2½ hours they were removed from the water baths and placed immediately into ice-cold water. After thorough cooling they were centrifuged, and the cells washed and resuspended and their power of reducing tetrathionate measured at 44° (Fig. 5 (*b*)). It can be seen that the rates of the reactions were then approximately linear, and that adaptation at 37° had been considerably more efficient than at 30° or 40°. It is of interest that, although approximately the same quantity of tetrathionate had been reduced by the suspensions at 37° and 40° after 2½ hours, 75 per cent more active enzyme was present in cells incubated at 37° compared to those at 40°.

A further experiment, in which temperatures closer to the apparent optimum for adaptation were employed, is summarized in Table III. In this instance, samples

TABLE III.—*Proportions of Active Enzyme Present in Cells of* Bact. paratyphosum B *After Adaptation at Different Temperatures.*

Temperature of adaptation.	Mean velocity of tetrathionate reduction at 44°—ml. of N/200 I_2 per ml. per 30 min.	
	After adaptation for 1 hour.	After adaptation for 2 hours.
33°	0·4	1·7
35°	1·1	4·0
37°	1·05	4·2
39°	0·4	1·5

were removed after 1 hours' and 2 hour's adaptation at 33°, 35°, 37° and 39°; after cooling and washing, the activities of the resuspended cells were measured at 44° as before. There was a fairly well-defined optimum adaptation temperature at about 35°–37°; in both the 1-hour and 2-hour samples the concentration of active enzyme (as judged by the mean velocities of reduction at 44°), after adaptation at 33° and 39°, was less than half the concentration after adaptation at 35° and 37°.

DISCUSSION.

Whilst these results do not throw much light upon the intrinsic nature of this enzyme adaptation phenomenon, they are an indication of the type of investigation that it is possible to carry out, and which can be used in the study of the effects of other environmental factors. The word "adaptation" has been used in preference to "production of enzyme," since we have been dealing with the end-result of what is probably a chain of several reactions, and have not simply been studying the growth of a single enzyme. Nevertheless it is fair to assume that the observed effects are true effects on the adaptation process, whatever that may prove to be.

It is, perhaps, worth pointing out that, although there is a fairly sharp optimum temperature for adaptation at about 36°, the actual amount of tetrathionate reduced during adaptation for the first 2 or 3 hours does not vary much between 30° and 40°. Thus the increased activity of free enzyme at temperatures above 37° compensates to a large extent for the less efficient adaptation, and the energy available from tetrathionate reduction does not vary as much as might be expected. This is analogous to the discovery by Gale and Epps (1942) that increased production of

certain enzymes by *E. coli* and *M. lysodeikticus* during growth at a pH removed from the optimum for enzyme activity maintains the effective cellular activity constant over a wide range of pH.

It is also of interest that the optimum temperature for the adaptation of tetrathionase in *Bact. paratyphosum B* is roughly the same as the optimum temperature for growth of the organism. It is possible that in a growing culture the two phenomena, growth and adaptation, are more closely linked than might at first appear from these results, which are based on the study of washed suspensions, incapable of proliferation.

SUMMARY.

The effect of temperature on the adaptation of "tetrathionase" in *Bact. paratyphosum B* has been studied, independently of cell growth, by the use of washed suspensions grown on nutrient agar. The optimum temperature for adaptation has been found to be 35–37°. At 44° the adaptation process is almost completely inhibited.

I am much indebted to Dr. Marjory Stephenson for her interest and encouragement, and for valuable suggestions concerning both the experimental work here reported and the preparation of this paper.

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THE BIOSYNTHESIS OF TRYPTOPHAN BY *BACT. TYPHOSUM*.

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Received for publication November 16, 1945.

FILDES, Gladstone and Knight (1933) deduced that when *Bact. typhosum* is able to grow on a medium containing only NH_3-nitrogen, it must have synthesized the tryptophan present in its substance from NH_3. Fildes (1940) argued that indole must be a stage in this synthetic process, because *Bact. typhosum*, or other bacteria which could not grow from NH_3, could often grow from indole and synthesize tryptophan. This argument has been generally accepted, but the actual synthesis of indole has not yet been proved.

The present paper deals with the synthesis of indole by *Bact. typhosum*, and its further use in the production of tryptophan.

Fildes (1938) showed that when *Bact. coli* was grown in the presence of indole acrylate, indole appeared in the culture. Washed suspensions of *Bact. coli*, however,

produced no indole from indole acrylate. He favoured the view that a synthetic process was involved, the indole acrylate being converted to tryptophan, which was the real source of the indole produced.

In the course of further work it has been found that this view is untenable.

Bact. typhosum normally grows in a mixture of amino acids only when tryptophan or indole is added (Fildes, 1940). In the present work another strain (NCTC 3390) was used with the same result. No indole was, of course, formed from tryptophan, and no indole was produced in cultures containing indole acrylate.

The same strain was now trained to grow without indole or tryptophan. It still was unable to produce indole on the addition of tryptophan, but, on the other hand, when indole acrylate was added, indole was produced.

The process of training had therefore changed the original 3390 into another, 3390*a*, which resembled *Bact. coli* in growing on the same NH_3 medium and producing indole when indole acrylate was present, but it still differed from *Bact. coli* in being unable to form indole from tryptophan.

Thus it could not be held in the case of 3390*a* that tryptophan synthesized from indole acrylate was the source of the indole produced.

Technique.

The culture medium used for the growth of untrained *Bact. typhosum* (3390) was an amino acid mixture composed as follows: aspartic acid 0·2 g., valine 0·15 g., leucine 0·15 g., glutamic acid 0·1 g., isoleucine 0·1 g., phenylalanine 0·1 g., lysine 0·1 g., glycine 0·05 g., proline 0·1 g., hydroxyproline 0·05 g., tyrosine 0·1 g., arginine 0·05 g., histidine 0·05 g.

With the exception of hydroxyproline, arginine and histidine, all were of synthetic origin. The natural substances were treated with norite and recrystallized three times. These amino acids were dissolved in tinned-copper distilled water containing KH_2PO_4 4·5 g. and N soda 26 c.c., made up to 700 c.c. at pH 7·6, distributed in 7 c.c. volumes in test-tubes, and autoclaved.

When setting up a test an addition was made to each tube as follows: water 0·25 c.c., M/2 glucose 0·25 c.c., anhydrous $MgSO_4$ M/123 0·1 c.c., Fe M/2500 0·1 c.c., cystine ≡ M/300 S in N/30 HCl 0·1 c.c., NaOH N/30 0·1 c.c., bacteria 0·1 c.c. This mixture was made in sufficient quantity from the sterile solutions and 1 c.c. added per tube.

All reagents were purified in the usual way. Fe was prepared as follows:

Analar citric acid, norited and recrystallized three times .	0·168 g.
Ferrous ammonium sulphate	0·156 g.
Water to	100 c.c.

For use dilute 1/10 and add 0·1 c.c. per tube.

The volume of each tube was brought up to 10 c.c. with water and any other additions required, e.g. indole, normally M/50,000 final; tryptophan (synthetic); indole acrylate. These were sterilized by glass Seitz filtration.

Molarities of synthetic amino acids are expressed in terms of one only of the optical isomers. The bacterial suspension added to each tube was taken from a 24-hr. agar slope in the case of 3390, standardized roughly against a suspension of barium sulphate, and so diluted with water that 0·1 c.c. contained about 10,000 organisms.

When the series of tubes was completed each was poured into a 50 c.c. Erlenmeyer flask and incubated in air containing 5 per cent CO_2.

The culture medium used for the trained *Bact. typhosum* (3390*a*) and *Bact. coli* (NCTC 86) was an ammonia medium: KH_2PO_4 4·5 g., NH_4Cl 0·5 g., $(NH_4)_2SO_4$ 0·5 g., M/2 lactate 50 c.c., Fe (undiluted stock solution as above) 1·0 c.c., anhydrous $MgSO_4$ 0·01 g., N soda 26 c.c., water to 700 c.c. pH 7·6. Distributed, etc., as above. At the time of setting up the test, water, glucose, cystine, soda and bacteria were added as above.

The bacterial suspension was made by diluting as above a 48-hour culture on the same medium. Glucose should not, however, be added to cultures to be maintained as stock. Other details were as above, or as indicated in previous papers from this department.

The experiments to be described were carried out as stated under aerobic conditions, but the main phenomena have been checked on the same media under anaerobic conditions using hydrogen and CO_2. Thus the relation of indole, tryptophan or serine to the growth of 3390, 3390*a* or *Bact. coli* and the inhibition and indole production in the presence of indole acrylate is qualitatively the same, though quantitative experiments have not been carried out.

THE EFFECT OF INDOLE ACRYLATE.

Exp. 1 shows an example of the effect of indole acrylate in inhibiting growth of 3390, 3390*a* and *Bact. coli* and on production of indole by 3390*a* and *Bact. coli*.

It is seen that with the three cultures indole acrylate inhibits growth for a variable length of time depending on the concentration, and that with 3390*a* and *Bact. coli* indole is produced when growth takes place. 3390 is inhibited to a greater extent than 3390*a* and *Bact. coli*.

The power to produce indole in the presence of indole acrylate, acquired by *Bact. typhosum*, as a result of being trained to grow without indole or tryptophan, is normal to many strains of *Bact. typhosum*, which can grow without indole or tryptophan spontaneously, as it is normal to *Bact. coli*.

Thus 3 out of 12 strains from the National Collection of Type Cultures grew without indole or tryptophan and produced indole in the presence of indole acrylate. Indeed, the training may take place spontaneously on some laboratory agar media, and for the present work the culture of 3390 has been dried to maintain it in the desired condition.

THE SOURCE OF THE INDOLE PRODUCED IN THE PRESENCE OF INDOLE ACRYLATE.

The indole may be produced (*a*) from breakdown of indole acrylate by a process equivalent to that by which *Bact. coli* produces indole from tryptophan, (*b*) by synthesis eventually from NH_3. The process has been followed quantitatively to throw light on this question.

Erlenmeyer flasks were used, capacity 2 l., and containing a shallow layer of 600 c.c. of the NH_3 medium *plus* indole acrylate and inoculated with 3390*a*. The flasks were filled with air + 5 per cent CO_2 and incubated. Samples were taken every 3 hours and the degree of growth measured by the Spekker photo-electric absorptiometer. For some purposes these readings were converted to bacteria per c.c. from a standard curve. The same samples were used for estimating indole by the distillation method of Woods (1935), using, however, Ehrlich's reagent and the Spekker, and converting to γ/c.c. from a standard curve. Controls showed that the method was of the order of 95 per cent accurate in estimating added indole; that bacteria alone produced no indole; that the indole reading was the same whether the whole culture or the clear supernatant was distilled; that indole acrylate did not yield indole on distillation.

EXPERIMENT 1.—*The Effect of Indole Acrylate.*

Experiment 1.—*The Effect of Indole Acrylate.*

Bact. typhosum, 3390, 3390*a*, and *Bact. coli* (NCTC 86); the two latter on NH_3 medium; 3390 on amino acids + indole. The degree of growth is indicated roughly by *plus* signs; tr. = trace. Indole is also indicated roughly by *plus* signs in brackets, the degree of production being judged roughly from the colour produced by adding to the whole culture an equal volume of Ehrlich's reagent. Readings must be taken at once owing to a later development of a green colour due to indole acrylate.

		Molarity of indole acrylate added M/—	Hours or days. 18.	25.	42.	49.	3.	4.	5.	6.
3390.										
1	Medium + M/50,000 indole	0	++(0)	+++	+++	+++	+++	+++	++	+++
2	Ditto	1,000	0	0	0	0	0	0	0	+
3	,,	2,000	0	0	0	0	0	0	0	+
4	,,	4,000	0	0	0	0	0	0	+	+
5	,,	8,000	0	0	0	0	0	+	+	+
6	,,	16,000	0	0	0	0	+	++	++	+
7	,,	32,000	0	0	tr.	+	++	++	++	+
	No indole found beyond that added.									
3390*a*.										
8	Medium alone	0	tr.	+++(0)	+++	+++(0)	+++	+++	++	+(0)
9	,, ,,	1,000	0	0	0	0	0(0)	tr.(tr.)	+(+)	++(+)
10	,, ,,	2,000	0	0	0	0	0(0)	+(+)	++(+)	+(++)
11	,, ,,	4,000	0	0	0	0	+(+)	++(+)	++(++)	+(+++)
12	,, ,,	8,000	0	0	0	tr.	+(+)	++(+)	++(++)	+(+++)
13	,, ,,	16,000	0	0	++(tr.)	++(++)	+++(+)	++(+)	++(+)	+(+)
14	,, ,,	32,000	0	0	++(+)	++(++)	+++(+)	++(+)	++(+)	+(+)
Bact. coli.										
15	Medium alone	0	0	tr.	+++(0)	+++	+++	+++	+++	+++(0)
16	,, ,,	1,000	0	0	0	0	0(0)	0	0	+(+)
17	,, ,,	2,000	0	0	0	0	0(0)	+(+)	++	++(+++)
18	,, ,,	4,000	0	0	0	0	+(+)	++(++)	++	++(+++)
19	,, ,,	8,000	0	0	0	tr.(+)	++(+++)	++(+++)	+++	+++(+++)
20	,, ,,	16,000	0	0	tr.(tr.)	++(+++)	+++(+++)	+++(+++)	+++	+++(+++)
21	,, ,,	32,000	0	0	+++(++)	+++(+++)	+++(+++)	+++(+++)	+++	+++(+++)

Experiment 3.—*Growth of* 3390a *with Tryptophan and Indole Acrylate*, M/9000.

Flask.			Hours. 16.	18.	24.	41.	64.
1	Medium + O		tr.(0)	+(0)	+++	+++	+++
2	,, + IA		0(0)	0	?(?)	+(++)	++(++++)
		M/					
3	,, + IA + T	36,000	++(0)	+++(0)	+++(0)	+++(0)	+++(0)
4	,, + IA + T	72,000	+	++(0)	+++(0)	+++(0)	+++(0)
5	,, + IA + T	144,000	tr.	+(0)	+++(0)	+++(0)	+++(0)
6	,, + IA + T	288,000	?	±(0)	++(?)	+++(+)	+++(+)
7	,, + IA + T	576,000	?	tr.(0)	+(tr.)	++(++)	+++(+++)
8	,, + IA + T	1,152,000	?	?(0)	±(+)	++(+++)	+++(+++)
9	,, + IA + T	2,304,000	?(0)	?(0)	tr.(+)	++(+++)	+++(+++)

Although a number of experiments have been made under similar conditions, the results have not been identical. Fig. 1 gives the results of three experiments, one in which the indole yield was highest, one lowest and one medium.

It will be seen that the production of indole is confined to the growing phase, as would be expected, since it has already been shown that none is produced by suspensions. It will also be noted that the indole curve rises smoothly to a peak which is higher than could be accounted for if the whole of the indole acrylate had been converted to indole. It is thus certain that some of the indole cannot have been derived from indole acrylate and, in the absence of any other source, must have been synthesized.

Actually the whole of the indole acrylate is not broken down, and therefore the proportion of indole produced by synthesis is still greater.

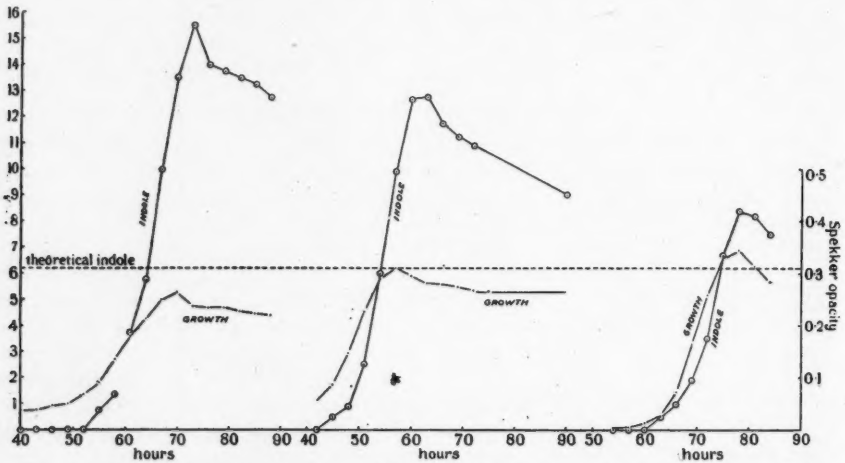


FIG. 1.—Quantitative experiments on indole production (μg./c.c.); three experiments—strain 3390*a* on NH_4 medium with M/18,000 indole acrylate.

An experiment was made in which growth, indole production and indole acrylate loss were all followed simultaneously. Indole acrylate was estimated by a method devised by Dr. Rydon. The supernatant from the centrifuged culture was made alkaline and extracted three times with equal volumes of petrol ether to remove indole, and an equal volume of Ehrlich's reagent was added. The mixture was heated at 100° for 10 minutes, and readings taken on the Spekker for comparison with a standard curve. A control showed that indole acrylate in an uninoculated flask remained constant during the course of the experiment. Fig. 2 shows that a relatively small fall (about 1/3) in the concentration of indole acrylate takes place during the growing phase, and that 4 times the equivalent concentration of indole is produced.

Whether the breakdown of indole acrylate leads to production of any indole is uncertain. It is noted that the production of indole does not coincide in time with the loss of indole acrylate, but this might be due to a production of indole at a later stage of indole acrylate breakdown. It is also noted that in all cases the production of indole rises smoothly to the peak. If the indole were produced from two sources, a break in the curve would be expected at the point at which one source failed.

The evidence is therefore against the supposition that a portion of the indole is derived from indole acrylate, and it is probable that the whole is the result of synthesis. In any case, the original deduction of Fildes (1940) was that *Bact. typhosum* must be capable of synthesizing indole, and the present results prove that this is correct.

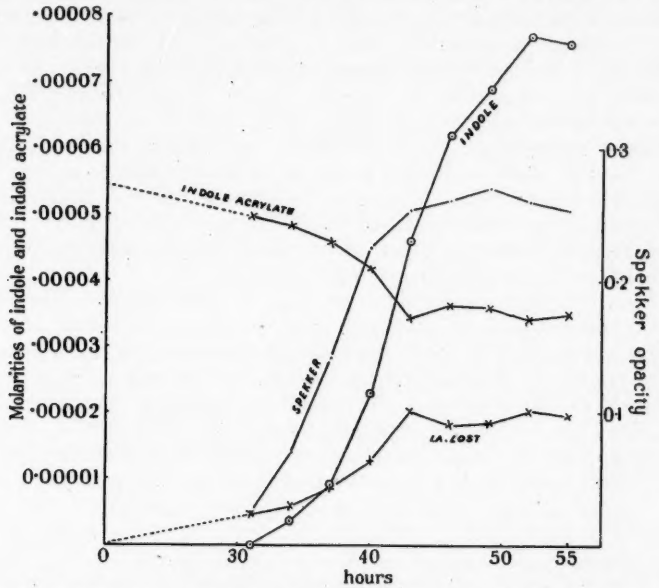


FIG. 2.—Relationship between growth, indole production and indole acrylate loss.

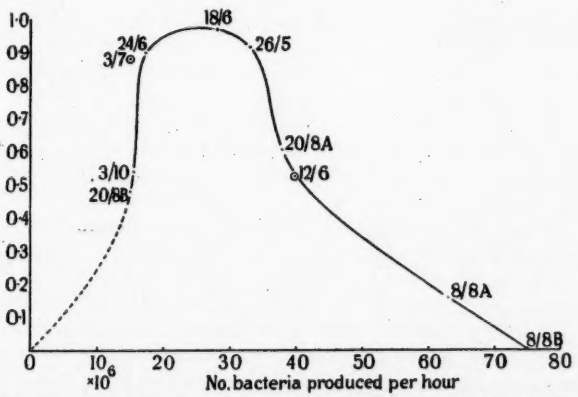


FIG. 3.—The rate of indole production is given as μg./c.c./hr., being calculated from the amount of indole produced above 1 μg./c.c. and the time taken. Similarly the Spekker opacity readings between 0·1 and the peak, converted to bacteria per c.c., and the time taken, give the number of bacteria produced per hour. All experiments were carried out with M/18,000 indole acrylic acid except those marked 12/6 and 3/7, for which M/28,000 was used. The NH_4 medium was used generally, but the amino acid medium in 8/8A, 8/8B, and 20/8B. Fe was omitted in 3/10 and 3/7.

In accordance with the conclusion of Fildes (1941) it is supposed that this synthesis of indole is a normal mechanism concerned with the eventual synthesis of tryptophan which is operated by bacteria which can grow from NH_3. If this power of synthesis and of growth is lacking, as in 3390, indole must be provided. The synthesis by *Bact. coli* or 3390*a* is only made apparent by the use of an inhibitor, indole acrylic acid, which delays the further use of indole, thus causing it to accumulate.

Reference has been made to irregularity in the amounts of indole produced under similar conditions. In Fig. 3 data from all experiments are taken, the rate of indole production being plotted against the rate of growth. It will be seen that high yields of indole are only obtained in cultures growing at a moderate rate.

A reasonable explanation of this result is that cultures which grow unusually slowly do so because their synthetic power is unusually depressed, for reasons unconnected with indole acrylate. This may be due to the individual bacteria being "sluggish" at the time of inoculation, or to the chance occurrence of an unusually high death-rate in the inoculum. The depression in the synthetic power of the culture as a whole is reflected in a poor yield of indole.

If depression of growth is absolute, as in a suspension, no synthesis takes place and no indole is produced.

At moderate rates of growth synthetic power is normal, but growth is impeded by a partial prevention of the further use of indole. Increasing rapidity of growth reflects a failure in the inhibition by indole acrylate and, therefore, an increased further use of indole and a decreased accumulation. The fastest rates of growth imply that inhibition by indole acrylate was ineffective, and that the indole was utilized when synthesized as in a normal culture.

EXPERIMENT 2.—*Growth of* 3390 *with Tryptophan* (*T*), *Indole Acrylate,* M/18,000 (*IA*) *and Indole,* M/50,000 (*I*).

Flask.				Hours. 18.	46.	52.
1	Amino acid medium without indole	+ 0		0	0	0
			M/			
2	Ditto	+ T	50,000	+++	+++	+++
3	,,	+ T	100,000	+++	+++	+++
4	,,	+ T	200,000	+++	+++	+++
5	,,	+ T	400,000	++	++	++
6	,,	+ T	800,000	+	+	+±
7	,,	+ T	1,600,000	±	±	+
8	,,	+ T	50,000 + IA	+++	+++	+++
9	,,	+ T	100,000 + IA	+++	+++	+++
10	,,	+ T	200,000 + IA	+++	+++	+++
11	,,	+ T	400,000 + IA	++	++	++
12	,,	+ T	800,000 + IA	+	+	+±
13	,,	+ T	1,600,000 + IA	±	±	+
14	,,	0	+ IA	0	0	0
15	,,	+ T	50,000 + IA + I	+++	+++	+++
16	,,	+ T	100,000 + IA + I	+++	+++	+++
17	,,	+ T	200,000 + IA + I	+++	+++	+++
18	,,	+ T	400,000 + IA + I	++	+++	+++
19	,,	+ T	800,000 + IA + I	+	+++	+++
20	,,	+ T	1,600,000 + IA + I	±	+++	+++
21	,,	0	0 + I	+++	+++	+++
22	,,	0	+ +	0	±	+

THE EFFECT OF TRYPTOPHAN.

Further experiments have been carried out to show that synthesized indole is, in fact, associated with the synthesis of tryptophan. Exp. 2 shows the effect on cultures of 3390 of tryptophan, indole acrylate and indole. It is noted that:

(*a*) Tryptophan allows growth to take place and, where it is the limiting factor, in proportion to the amount of tryptophan added. The same effect (not shown) is obtained with indole, though somewhat higher concentrations are necessary.

(*b*) A concentration of indole acrylate which is inhibitory in the presence of indole (flask 22) has no inhibitory action in the presence of tryptophan (flasks 8–13, compared with 2–7).

(*c*) When tryptophan, indole and indole acrylate are present, the initial growth is equivalent to the growth with tryptophan and indole acrylate (flasks 15–20 compared with 8–13), but later the flasks in which tryptophan is limiting (18–20) grow up equivalently to the growth produced by indole alone (flask 21 compared with 20).

It is concluded that indole acrylate has no inhibitory action on growth due to tryptophan, but only on growth due to indole. When both are present, the initial growth is due entirely to tryptophan, whereas later growth in those flasks in which growth is not already maximal is due to indole.

At no stage did the cultures contain indole beyond that added. These results are in accordance with the view of Fildes (1941) that indole acrylate inhibits the further use of indole in the synthesis of tryptophan.

Exp. 3 shows the effect of tryptophan and indole acrylate on the growth of 3390*a*. It will be seen that the growth in this experiment is the same as that of Exp. 2. Thus the early growth is in proportion to the tryptophan added, and later growth takes place similar to that due to added indole in Exp. 2. In Exp. 3, however, no indole was added, but indole has been synthesized in those flasks in which later growth has occurred.

It is concluded in the case of 3390*a* that the later growth is due to indole synthesized, whereas with 3390 it is due to indole added. Both 3390 and 3390*a* are totally unaffected by indole acrylate when tryptophan is present; both are inhibited by indole acrylate when tryptophan is not present or when tryptophan added has been expended. This inhibition is eventually overcome, and then growth proceeds in the one case by means of indole added and in the other by indole synthesized.

The significance of the growth of 3390*a* without indole synthesis in M/144,000 tryptophan is that this is the lowest concentration of tryptophan which will allow full growth under the conditions of the test. The figure is similar (cf. Exp. 2) in the absence of indole acrylate. It is also the same when three different concentrations of indole acrylate are used, e.g., M/4500, 9000 and 18,000 (not shown).

This result is in accord with the view that when an essential metabolite is added to a culture containing an inhibitor of its synthesis, full growth takes place in the same concentrations of the metabolite as would be effective without the inhibitor, i.e. in accord with the view of Fildes (1941) that indole acrylate prevents the synthesis of tryptophan by an action exerted on a stage of the synthesis between indole and tryptophan.

THE EFFECT OF SERINE.

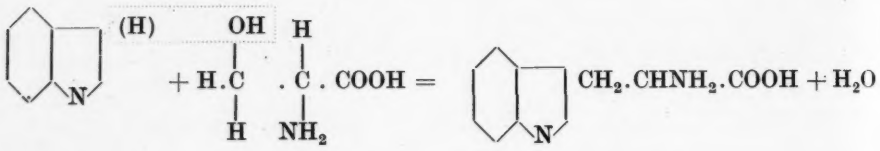
Fildes (1941) argued that, if the synthesis of tryptophan was achieved in one step from indole, the inhibitory effect of indole acrylate should be at least

partially overcome by increasing the concentration of indole. This was not the case.

He therefore assumed the existence of an intermediate X between indole and tryptophan, and supposed that indole acrylate interfered with the stage X——→ tryptophan.

In the meantime the subject has been illuminated by the work of Tatum and Bonner (1944). They have shown that a suspension of *Neurospora* can synthesize tryptophan in a simple solution of indole and serine.

They also referred very shortly to experiments with *Bact. coli*, which in their view supported the idea that *Bact. coli* also can combine indole and serine to form tryptophan according to the equation :



Tatum and Bonner's reference to their *Bact. coli* experiments is quoted in full :

"We have also performed a few experiments in which indole alone and indole with serine were incubated with coli suspensions. The results showed clearly that serine has an effect on the disappearance of indole similar to that in *Neurospora*. Colorimetric tests for tryptophan after 24 hours' incubation indicated that some tryptophan had been synthesized from indole in the presence of serine."

It was plain that if the final stage in the synthesis of tryptophan consisted of a coupling of indole and serine, Fildes' (1941) intermediate X need no longer be postulated. It would become a question of an intermediate *process*, which might not be affected by alteration in the concentration of one of the components.

EXPERIMENT 4.—*The Effect of Serine on Inhibition by Indole Acrylate.*

Strain 3390. Amino acid medium + M/50,000 indole. Indole acrylate M/4000. Synthetic serine was used, the molarities referring to the active isomer.

Flask.		Serine. M/	Hours. 18.	21.	27.	46.	66.	90.	114.
1	Medium + O	0	++	+++	+++	+++	+++	+++	++
2	,, + IA	0	0	0	0	0	0	tr.	+
3	,, + IA	100	0	0	tr.	+++	+++	+++	+++
4	,, + IA	200	0	0	tr.	+++	+++	+++	+++
5	,, + IA	400	0	0	tr.	+++	+++	+++	+++
6	,, + IA	800	tr.	+	++	+++	+++	+++	+++
7	,, + IA	1,600	0	tr.	+	↓	+++	+++	+++
8	,, + IA	3,200	0	tr.	+	↓	↓	+++	+++
9	,, + IA	6,400	0	0	0	↓	↓	↓	+++
10	,, + IA	12,800	0	0	0	↓	↓	↓	+++
11	,, + IA	25,600	0	0	0	tr.	++	↓	↓
12	,, + IA	51,200	0	0	0	?	+	↓	↓
13	,, + IA	102,400	0	0	0	0	+	++	↓
14	,, + IA	204,800	0	0	0	0	+	+	↓
15	,, + IA	409,600	0	0	0	0	tr.	+	++

Vertical arrows indicate a graduated fall in opacity.

EXPERIMENT 5.—*The Effect of Serine on Inhibition by Indole Acrylate and on Indole Synthesis.*

Strain 3390*a*—amino acid medium used without indole, because serine is too highly inhibitory on the NH_3 medium. Indole acrylate and serine as in Experiment 4.

Flask.		Serine.	Hours. 17.	24.	40.	65.
		M/—				
1	Medium + O	0	+++	+++(0)	+++(0)	+++
2	„ + IA	0	0	0	tr.(tr.)	+(+++)
3	„ + IA	100	0	++(0)	+++(0)	+++(0)
4	„ + IA	200	0	++(0)	+++(0)	+++(0)
5	„ + IA	400	0	++(0)	+++(tr.)	+++(tr.)
6	„ + IA	800	0	++(0)	+++(++)	+++(++)
7	„ + IA	1,600	±	++(+)	(+++)	+++(+++)
8	„ + IA	3,200	tr.	++(+)	(+++)	(+++)
9	„ + IA	6,400	tr.	++(+)	(+++)	(+++)
10	„ + IA	12,800	tr.	+(+)	(+++)	(+++)
11	„ + IA	25,600	tr.	tr.(+)	++(+++)	++(+++)

Effect of serine on inhibition by indole acrylate.

Experiments were carried out to show whether serine affected the inhibition by indole acrylate. Experiments 4 and 5 indicate the results. With both 3390 and 3390*a*, the inhibition is so much reduced that it seems probable that if the higher concentrations of serine were not inhibitory (as are many amino acids), they would have allowed growth as rapidly as occurred in the control without indole acrylate. At first the effect of serine is quantitative, especially with 3390, but later the lower concentrations grow up owing, probably, to an increase in serine concentration by synthesis. In the case of 3390*a* indole appears in the cultures except in concentrations of serine about M/400. It may thus be concluded that indole acrylate has no effect either in inhibiting growth or in causing the accumulation of synthesized indole when sufficient serine is present. This effect of serine was not shown by alanine, threonine, cysteine, β-hydroxypropionate or pyruvate.

EXPERIMENT 6.—*Relationship Between the Concentrations of Indole Acrylate and Serine.*

Details as in Experiment 5.

Flask.		IA.	Serine.	Hours. 17.	25.	41.	65.
		M/	M/				
1	Medium +	0	0	+++(0)	+++	+++	+++(0)
2	„ +	1,000	0	0	0	0	+(tr.)
3	„ +	1,000	90	0	0	+++(0)	+++(0)
4	„ +	1,000	360	0	0	+++(tr.)	+++(0)
5	„ +	1,000	1,440	0	0	++(+)	++(+)
6	„ +	4,000	0	0	tr.(tr.)	+(++)	++(++)
7	„ +	4,000	90	tr.(0)	+++(0)	+++(0)	+++(0)
8	„ +	4,000	360	tr.(0)	+++(0)	+++(0)	+++(0)
9	„ +	4,000	1,440	0	+(0)	++(++)	++(++)
10	„ +	16,000	0	+(0)	++(++)	++(++)	++(++)
11	„ +	16,000	90	+++(0)	+++(0)	+++(0)	+++(0)
12	„ +	16,000	360	+++(0)	+++(tr.)	+++(0)	+++(0)
13	„ +	16,000	1,440	++(0)	++(++)	++(++)	++(++)
14	„	0	90	+++(0)	..	..	..
15	„	0	360	+++(0)	..	..	..
16	„	0	1,440	+++(0)	..	..	..

Exp. 6 shows that the lowest concentration of serine which allows full growth without indole production, i.e. which is unaffected by indole acrylate, is the same whatever the concentration of indole acrylate, and the same argument might be used as in the case of tryptophan—that this is the lowest concentration of serine which gives full growth without an inhibitor, and therefore that indole acrylate is inhibiting the synthesis of serine. This argument, however, is inapplicable, because full growth takes place without any added serine at all. Added serine is necessary only in the presence of the inhibitor. It might also be argued that because there is no relationship between the concentration of the inhibitor and that of the substrate, this cannot be a case of a competition between inhibitor and substrate for the use of an enzyme concerned in coupling indole and serine. But this argument is only available in such cases as those in which Woods (1940) used it, i.e., when inhibition is absolute so that the concentration of enzyme remains constant, and when the inhibitor and substrate are stable. In the present case the enzyme concentration is progressively increasing during growth ; the inhibitor is decreasing (*cf.* Fig. 2), and the substrate serine is rapidly decreasing (*cf.* Hills, 1940). The theoretical concentration of serine necessary for the synthesis of sufficient tryptophan to give full growth is about M/144,000. To achieve this it is necessary to add 400 times as much because of its rapid destruction or utilization in other ways. Under these circumstances it is impossible to show quantitative relationships between the factors involved.

If indole acrylate exerts its effect by inhibiting the synthesis of serine, it could only do so by virtue of its side chain, and another substance with the same side chain might operate in the same way.

The work of Block and Erlenmeyer (1942) suggests that it does. They claimed that β[naphthyl (1)] acrylic acid inhibited *Bact. coli*, and that this inhibition was reversed by tryptophan. A study of the protocols, however, discloses that the amount of growth in the control without inhibitor was insignificant, and therefore the action of the inhibitor insignificant. Tryptophan markedly stimulated growth with or without inhibitor. The experiments cannot therefore be used in the present argument.

Acrylic acid itself in concentrations as high as M/100 will inhibit *Bact. coli*, but this is not counteracted by tryptophan, and so must be due to a cause different from that under consideration. β-phenyl acrylate (cinnamate) does not inhibit in any concentration tested.

Thus by exclusion it seems more probable that the effect of indole acrylic acid depends upon its indole component and its side chain, i.e. that it exerts an action both on indole and on serine, and that this action is an interference with the dehydrative condensation of these two substances which is the normal mechanism for the synthesis of tryptophan by *Bact. typhosum*, as has been proved to be the case in *Neurospora* by Tatum and Bonner.

THE DIRECT SYNTHESIS OF TRYPTOPHAN.

Attempts were now made to confirm the crucial experiments of Tatum and Bonner on the production of tryptophan by bacteria acting on solutions of indole and serine.

Tatum and Bonner digested washed suspensions of *Bact. coli* with constant concentrations of indole and rising concentrations of serine, and showed that indole disappeared from the mixture in proportion to the amount of serine added. In the mixtures from which indole had disappeared they detected " some tryptophan " by colorimetric methods.

These experiments have been repeated with *Bact. coli*, but no loss of indole was

detected within the limits of accuracy of the distillation technique used. It was thought possible that this failure to confirm Tatum and Bonner's results might have been due to the use of a strain of *Bact. coli* which was more active in breaking down tryptophan. When using *Bact. coli* it is clear that any tryptophan produced by condensation of indole and serine is liable to be converted immediately into an equivalent of indole, and therefore with no loss of indole in the mixture. We therefore resorted to *Bact. typhosum* 3390.

EXPERIMENT 7.—*Direct Synthesis of Tryptophan.*

Five attempts have been made to show a loss of indole under the influence of serine and a synthesis of tryptophan. In 3 experiments, $2\frac{1}{2}$ equivalents of serine were used, and in 2, 13 equivalents.

The procedure in 4 cases was to grow 3390 for 16 hours, and in 1 case for 6 hours, on tryptic-meat agar, and wash off the culture in water to a volume of 100 c.c. of a suspension containing about 10^{10} bacteria per c.c. The bacteria were deliberately not washed. This suspension was added to 100 c.c. solution containing KH_2PO_4, $MgSO_4$, Fe and glucose to give the final concentrations as used in cultures and also final concentrations of M/1300 indole and the selected concentration of serine. The mixture in a shallow layer in an Erlenmeyer flask was incubated at 37° in air and CO_2. A control flask without serine was also incubated.

Samples were taken at once and at intervals of 3 hours or longer during 24 hours or longer to 3 days. On each occasion indole was estimated by the distillation method and tryptophan by Woods' (1935) method.

The results of all experiments were negative in that no obviously significant loss of indole occurred and no tryptophan was produced.

The following result with 13 equivalents of serine and 16-hour bacteria was the most significant:

Sample.	Hours.	Indole μg./c.c.	
		With serine.	Without serine.
1	0	90	89
2	6	86·2	88
3	23	80·0	88
4	30	82·0	86
5	47	83·0	84·2
6	54	82·0	90
7	71	84·0	87

On this occasion the indole estimations were somewhat variable and therefore of less significance. Usually the results showed a variation no greater than 2 μg./c.c. with or without serine.

These figures, at most, could indicate that 7–8 μg./c.c. indole had disappeared, i.e. about 8 per cent of that present. This represents about 13 μg./c.c. tryptophan possibly produced. The tryptophan estimations showed tryptophan in all samples in concentrations too low to measure by the Spekker, i.e. of the order of 4 μg./c.c. The presence of tryptophan is clearly accounted for by the unwashed suspension.

The five experiments described as Exp. 7 failed to demonstrate any significant loss of indole or any production of tryptophan.

Direct proof of the condensation of indole and serine to produce tryptophan by *Bact. coli* or by *Bact. typhosum* is therefore lacking in this work. It is not, however,

felt that this failure is fatal to a conclusion that this reaction does in fact take place.

Catabolic processes actuated by enzymes in bacterial suspensions with liberation of energy have been well analysed, and in some cases can be reproduced by the isolated enzyme. The conditions under which bacterial enzymes carry out synthetic processes with absorption of energy are less well known, and can seldom yet be reproduced artificially, except in the complex process of growth. It seems probable that a bacterial synthesis of tryptophan in excess of growth requirements will be demonstrated most clearly when a partial inhibitor of its further use has been found.

SUMMARY AND CONCLUSIONS.

1. The synthesis of free indole by bacteria has been demonstrated.
2. The indole synthesized is a component in the synthesis of tryptophan.
3. The stage indole——→tryptophan is probably effected by dehydrative condensation of indole with serine as occurs with *Neurospora* (Tatum and Bonner).

I have to thank Dr. H. N. Rydon for synthesizing indole acrylic acid and other help, Dr. D. Herbert for carrying out some of the estimations of indole, and both Dr. D. D. Woods and Dr. Herbert for criticisms during the course of the work.

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VOL. XXVI. No. 6] [DECEMBER, 1945

THE BRITISH JOURNAL OF EXPERIMENTAL PATHOLOGY

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THE BRITISH JOURNAL OF EXPERIMENTAL PATHOLOGY.

VOL. XXVI, No. 6. DECEMBER, 1945.

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Date of Publication.—The Journal is published bi-monthly, in the middle of February, April, June, August, October, and December.

Rapidity of Publication.—All papers accepted for publication will be printed, so far as possible, in the next following issue of the Journal, if received before the middle of the month preceding publication. Long papers, or papers with illustrations, may, however, be delayed if not received earlier.

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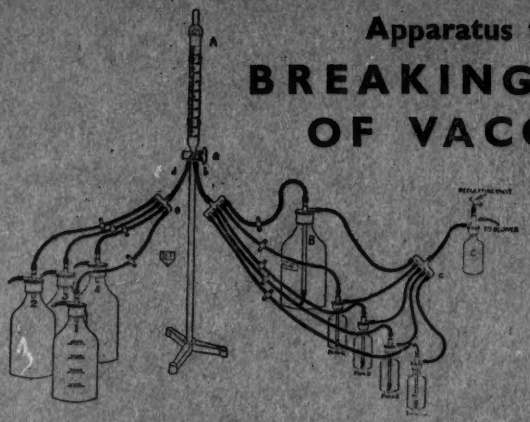

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TABLE VIII.—*Nitratase Adaptation Without Growth by Washed Suspension of Salm. Paratyphi B.*

	Before adaptation.	After 2½ hours' adaptation at 35° with nitrate and mannitol.
Bacterial N	0·39 mg./ml.	0·38 mg./ml.
Opacity (drum reading after diluting 1 : 20)	0·212	0·211
Nitratase activity at 35° with formate as H-donator; conc. nitrite formed in M × 10^{-5} per hour . . .	4·4	25·8

for supposing that the phenomenon of adaptation is much different in *Salm. paratyphi B* from that in " 1433."

DISCUSSION.

The adaptation of nitratase by washed suspensions of bacteria appears to be essentially similar to that of tetrathionase. There is, perhaps, less relative increase in nitratase under comparable conditions, but this may be due to an apparently greater initial activity in unadapted cells. The adaptation of bacterial enzymes in the absence of, or at least independently of cell growth is probably a phenomenon more widespread than is commonly realized. It is perhaps surprising that it has not previously been reported for an enzyme-system as well known as nitratase. In this connection it is interesting to refer again to Stickland's original paper (1931), where he claims that the rate of nitrate reduction by dilute washed suspensions of *Bact. coli* is constant for at least 90 minutes. This is not surprising, since under such conditions little or no adaptation might be expected to occur whatever H-donator is used. He goes on to point out, however, that with a thick suspension the whole course of the reduction of M/50 nitrate to nitrite can be followed over a period of 5 hours. The explanation of why the phenomenon of adaptation was missed is not far to seek—although a study of the published figure does actually show an upwardly curved graph indicating increased nitratase activity of about 100 per cent which is not commented upon. The reason is no doubt because lactate was used as the H-donator and the reaction followed at 40°. It has previously been pointed out that lactate is a relatively inefficient H-donator for adaptation, and, to judge from results obtained with " 1433 " and *Salm. paratyphi B*, 40° is almost certainly well above the optimum adaptation temperature. If the reaction had been followed at 35° with mannitol, it is most likely that the upward trend of the curve would have been too striking to have been ignored.

The enzyme-system nitratase, like tetrathionase, cannot of course yet be regarded as a single enzyme; it depends for its action upon the activation of H-donators by other enzymes as well as upon the interaction of a H-carrier. For these and for other reasons already pointed out (Knox and Pollock, 1944) it is impossible to claim that increased nitratase activity necessarily represents the production in the cell of a single enzyme protein. But whether the hypothetical enzyme protein is visualized as being synthesized *de novo* or merely resulting from specific alteration of some indifferent cell protein (which is one of the suggestions

put forward by Spiegelman (1945) to explain enzyme adaptation), this still remains the most likely explanation. The complete specificity of tetrathionase and nitratase adaptation within the same cell suspension lends support to this hypothesis. If, for instance, nitratase adaptation depended largely upon coenzyme production or formation of a dehydrogenase rather than upon an enzyme specifically activating the reduction of nitrate, one might expect in addition some difference in the subsequent rate of reduction of tetrathionate which in almost every respect is a very similar process to reduction of nitrate. No such alteration occurs; cells adapted to nitrate reduce tetrathionate no more rapidly than untreated cells, and *vice versa*. Moreover, adaptation to either tetrathionate or nitrate in the presence of mannitol does not lead to any increased activity of mannitol dehydrogenase, as judged by the methylene-blue reduction test.

Both nitratase and tetrathionase adaptation curves are similar to those of the adaptation in washed suspensions of formic hydrogenlyase of *Bact. coli* (Stephenson and Stickland, 1932), yeast galactozymase (Schultz, Atkin and Frey, 1940; Spiegelman, 1945), and yeast glucozymase and melibiozymase (Spiegelman, 1945). In all these cases the beginning of adaptation is characterized by a rising rate of enzyme "formation" and the curves are not far removed from the exponential. It has been argued (Spiegelman, 1945) that this enzyme adaptation is due to the self-reproduction of either the enzyme protein itself or some protein or nucleo-protein precursor. Some such explanation would certainly appear to be necessary in order to understand the apparent self-perpetuation of the melibiozymase of haploid progeny of *S. cerevisiae–S. carlsbergensis* hybrids in the absence of the gene known to be originally responsible for its production (Spiegelman, Lindegren and Lindegren, 1945). The genetical approach to the problem has been considered in some detail by Spiegelman (1945) and Lindegren (1945). Lindegren (1945) goes so far as to equate the adaptive enzyme to his "cytogene," which he considers to be a self-reproducing cytoplasmic entity differing only from Darlington's "plasmagene" in being dependent upon the presence of a specific gene for its perpetuation in the absence of the specific substrate. Spiegelman and Lindegren's work has been done on nucleated yeast cells where genetical factors can be exactly determined, but these hypotheses, particularly in the case of bacteria, remain at present largely speculative.

SUMMARY.

1. Washed suspensions of a lactose-fermenting coliform organism ("1433") if incubated with mannitol and nitrate at 35° show a gradually increasing rate of reduction of nitrate to nitrite over a period of 2 to 4 hours.

2. The increase in nitratase activity, which may be up to 20 times (or more) that of the untreated suspension, is retained after repeated washing, and takes place without any increase in bacterial N, opacity measurements, or number of viable organisms.

3. Mannitol, lactate and formate can act as efficient H-donators for nitrate reduction by adapted cells, but no adaptation will occur with formate, and only a little with lactate. Adaptation with mannitol is by far the most rapid.

4. Little or no adaptation occurs at 44° or above.

5. Adaptation of nitratase appears to be closely analogous to that of tetrathionase.

6. Nitratase adaptation in washed suspensions is specific to nitrate and will not occur in its absence. Adaptation to both nitrate and tetrathionate can be induced independently in the same bacterial suspension by simultaneous or successive treatment with the two substrates.

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